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Multifaceted Regulation of Peripheral T Cell Tolerance and Autoimmunity by FOXP3+ T Regulatory Cells: A Dissertation

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Multifaceted Regulation of Peripheral T cell Tolerance and Autoimmunity by FOXP3^{+} T Regulatory Cells

A Dissertation Presented

By

Nitya Jain

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Immunology and Virology

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DEDICATION

This thesis is dedicated to Dr. Cynthia Chambers.

‘*You were there at the beginning,*

Then you went away,

But your spirit guided me

And, I knew you would be there

To see this through’

Thank you.

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ABSTRACT

Adaptive immunity requires T cell responses to foreign pathogens to be counterbalanced with the need to limit collateral destruction of the host's own tissues. Further, the presence of a substantial pool of lymphocytes capable of recognizing self-antigen in the periphery poses a threat to the maintenance of peripheral tolerance and prevention of autoimmunity. Regulatory T cells (Treg) that can suppress potentially self-reactive T cells are critical regulators of peripheral tolerance as well as initiation of immune responses. Treg cells employ several context-dependent mechanisms to establish regulation. In this thesis, we describe two distinct pathways of regulation used by Treg cells involving negative costimulation by CTLA-4 and immunomodulation by the morphogen, TGFβ.

CTLA-4 is a co-inhibitory receptor on T cells essential for maintaining T cell homeostasis and tolerance to self. CTLA-4 expression is induced in conventional T cells following activation, whereas it is constitutively expressed in regulatory $FOXP3^{+}CD4^{+}$ regulatory T cells. Mice lacking CTLA-4 develop an early onset, fatal breakdown in T cell tolerance. Whether this autoimmune disease occurs because of the loss of CTLA-4 function in regulatory T cells, conventional T cells, or both, is not known. We present evidence here that in addition to a critical CTLA-4 function in regulatory T cells, CTLA-4 in conventional T cells is also necessary for controlling the consequences of abnormal T cell activation. CTLA-4 expression in activated conventional T cells only *in vivo* is unable to compensate for the impaired function of CTLA-4-less regulatory T cells that

results in systemic lymphoproliferation, but it can prevent the aberrantly activated T cells from infiltrating and fatally damaging non-lymphoid tissues. These results demonstrate that CTLA-4 has a dual function in maintaining T cell homeostasis: CTLA-4 in regulatory T cells inhibits inappropriate naïve T cell activation and CTLA-4 in conventional T cells can prevent the harmful accumulation of inappropriately activated pathogenic T cells in vital organs.

In addition, we have identified *Disabled-2* (*Dab2*), a TGFβ signaling intermediate, as a FOXP3 target gene that is expressed exclusively in Treg cells and is critical for *in vitro* and *in vivo* regulation by Treg cells. During T cell development, DAB2 is also expressed in a *Foxp3*-independent manner in thymic precursor cells, and acts as a sensor of TGFβ signals that is required for programming normal TGFβ responsiveness in T cell progenies. Naïve $CD4^+$ T cells that differentiate from *Dab2*-deficient precursors favor Th17 cell generation at the expense of $FOXP3^+$ Treg cells as a result of altered sensitivity to TGFβ. Importantly, retinoic acid can restore TGFβ signaling capacity of naïve $CD4^+$ T cells generated from Dab2-deficient precursors, emphasizing the cooperative nature of retinoic acid and TGFβ signaling pathways in promoting Treg cell development and maintenance.

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CHAPTER 1

INTRODUCTION

There is no need to be a doctor or a scientist to wonder why the human body is capable of resisting so many harmful agents in the course of everyday life.

Ilya Metchnikov, Nobel lecture, 1908.

Disease and Immunity

The Merriam-Webster dictionary defines disease as "*a condition of the living animal or plant body or of one of its parts that impairs its normal functioning and is typically manifested by distinguishing signs and symptoms*". Diseases can arise due to a myriad of factors including genetic or developmental defects, infections, poisons, nutritional deficiency or imbalance, toxicity, or unfavorable environmental conditions. The organism's defense against these factors is called immunity (Latin: *immunis*) and the collection of mechanisms within an organism that function to provide this protection is called the immune system.

Over the course of history, there have been several indications of practices that were used to prevent and fight diseases. A formal demonstration of deliberate vaccination came in the late 18th century when Edward Jenner successfully used the harmless cowpox virus to immunize people against the smallpox causing vaccinia virus. However, most of the conceptual framework of the immune system was laid down much later in the 19th and early 20th century. In the words of Alfred Tauber, '*Immunology was born in the controversies of that fresh announcement that no*

species, including our own, was a static entity; all were subject to change as a result of the vicissitudes of time and happenstance' (1). Tauber refers, of course, to Charles Darwin's *On the Origin of Species,* which was published in 1859 and generated intense furor in the scientific community. His evolutionary theory proposed an ever-changing species in which favorable changes were inherited in successive generations of a reproducing organism. While this theory was vigorously opposed by several scientific and religious factions, it became apparent that the schema of an organism was not static, but was always adapting and evolving. In the backdrop of this revolutionary idea, several key discoveries were made that changed the way people thought about disease and immunity.

At the time, two lines of scientific inquiry existed- one that investigated the role of microorganisms as the primary cause of disease and the other that investigated host responses to these infectious agents. The former group was lead by Louis Pasteur who had decisively discarded the prevalent idea of 'spontaneous generation' and demonstrated, along with Robert Koch, that microorganisms were the agents responsible for disease. Medical practices were revolutionized by the contributions of Pasteur, Koch, Alexander Fleming and Joseph Lister to name a few, and the cause-effect relationship between microbes and diseases was firmly established.

Ellie Metchnikoff, a Russian zoologist, was in the forefront of the second faction. Shortly after the publication of *Origin*, he performed the famous Messina experiments, a classic example of cellular immunology in which phagocytes from a starfish larvae punctured with a rose thorn would attempt to protect the larvae by

engulfing the intruding thorn tip. This was the first demonstration of a host cell population that was equipped to attack foreign potentially infectious agents and provide protective immunity. Around the same time, Paul Ehrlich and Emil von Behring, working with diphtheria toxin, elaborated the theory of 'antigenicity', which led to the idea that only certain substances can elicit the production of antibodies. Ehrlich also predicted the phenomenon of *'horror autotoxicus*' or autoimmunity wherein, the host can, under certain conditions, make substances that can harm itself. Thus, by the beginning of the 20th century, two situations were defined that formed the fundamentals of immunity: '*host identity*' or the ability of the host to distinguish between 'self' and 'non-self' and '*host integrity*' or the ability of the host to counteract insults and injury.

Herein, I elaborate on these two concepts, beginning with a brief discussion on the 'immune self' and mechanisms of establishing self-tolerance, followed by a detailed review of the role of T regulatory cells in maintaining tolerance in the periphery. Finally, I discuss the role of cytotoxic T lymphocyte antigen-4 (CTLA-4) and transforming growth factor β (TGFβ) as important effectors of immune regulation and T regulatory cell function.

Immune Self and Tolerance

In the early 1940's, immunologists, working with diverse experimental systems, introduced the concept of self-tolerance, which has since become one of the organizing concepts of Immunology. The term 'self' was first coined by Frank Macfarlane Burnet, a virologist, who postulated that the 'self' of the host body was

actively defined during its embryogenesis through interactions between cells of the immune system and other cells within the embryo (2). During this process, the developing immune system is taught to be tolerant to self, such that, upon antigenic challenge, it could mount a potent response against foreign, non-self antigen and leave host self-tissue untouched. This was experimentally demonstrated by Peter Medawar, Rupert Billingham and Leslie Brent who showed that neonatal or *in utero* injections of cells from mice of one strain (A) into a second strain (B) tolerized the strain B mice to donor A antigens when challenged in adulthood (3). Interestingly, Owen had already observed this phenomenon of tolerance when he was studying binovular twin cattle that shared a common placenta (4).

While there was a consensus among immunologists about the existence of self, the 'nature' of self was and still remains disputed. The cluster of genes that form the Major Histocompatibility Complex (MHC) can be considered as one of the main signatures of self. Pioneering work by Gorer and Snell identified these antigens as being responsible for the rejection of foreign engrafted tissue (5). However, while the MHC may be one identity of self, it is probably not *the* self itself. Classical experiments by Peter Doherty and Rolf Zinkernagel showed that the MHC was not the solution to the dilemma of self, but was an additional complexity to the immune system, because now the lymphoid cells did not simply have to deal with discriminating between self and non-self; it also had to distinguish between self and self plus MHC (6).

The current dogma is that the 'immune self' is not a static entity, but evolves during the life of an individual and the life of a species. While this idea challenges the simple reductionist approach that a single genetic criterion defines self, it serves better to explain the constant changes in an organism's identity over ontogeny and evolution. More recent developments in the field have led immunologists to reconsider the necessity of self-non-self discrimination by the immune system. One emerging idea is that 'dominant' mechanisms of tolerance operate constantly and therefore, the immune system does not *need* to bother with self-recognition. Polly Matzinger is the protagonist of another school of thought, which completely discards the requirement of self-non-self discrimination for proper immune function. Her thesis instead proposes a "danger" model, wherein the host, instead of discriminating between what is self and what is foreign, now responds to the presence of 'danger'-namely antigens secreted by damaged host tissues (7). A distinguishing feature of this theory is that the infectious agents themselves are not recognized as 'dangerous', but rather, the host tissues that are damaged in the process of infection, transmit the signals of damage.

Regardless of what self is, it is clear that the induction of self-tolerance requires the presence of antigen-sensitive cells that can 'recognize' antigen, a process which later impairs the cells' capacity to respond to that antigen (8). Of the cells of the immune system, T and B lymphocytes have the ability for specific recognition of an enormous array of antigens by virtue of their expression of antigen-specific receptors, and are therefore, the key effectors of self-tolerance. The subsequent

discussions are restricted to the role of T cells in mediating and establishing immune tolerance.

T cell tolerance

The T cell receptor (TCR) was identified in the late 1980's using clone-specific monoclonal antibodies to T cell lymphomas and hybridomas (9). TCRs are generated by random genetic rearrangements of the V, D and J elements that can give rise to up to 10^9 V-D-J genes in mice (10-12). The peripheral T cell repertoire is represented by an estimated $25x10^6$ TCRs (13). Due to this enormous diversity in the recognition capabilities of the TCR and the plasticity of antigen recognition by these TCRs, mechanisms must exist that protect the host from damage by self-reactive T cells. These mechanisms of T cell tolerance can be divided into two categories: central tolerance, operating in the thymus, and peripheral tolerance, operating in peripheral tissue and lymphoid organs.

Central tolerance broadly refers to the purging of the self-reactive repertoire as T cells develop in the thymus. T lymphocytes arise from multi-potent precursors in the bone marrow that are called haematopoietic stem cells (HSCs). Committed lymphoid progenitors migrate from the bone marrow to the thymus via the blood circulation. The earliest precursors to seed the thymus are the early T cell progenitors called ETPs, which are phenotypically characterized as Lin^{low} (Lin: lineage markers), KIT^{hi} (Kit: receptor for the cytokine SCF, or stem cell factor), IL-$7R^+$ and $CD25^-$ (IL-2Rα) (14). They have multi-lineage potential and can differentiate into B cells, T cells, NK cells, and myeloid cells. ETPs then undergo ordered differentiation to

become DN2 (double negative, $CD4^-CD8^-CD25^+CD44^+$), DN3 ($CD4^-CD8^-CD25^+CD44^-$) and DN4 ($CD4^-CD8^-CD25^-CD44^-$) cells (15) (**Figure I-1**). Myeloid, B and NK cell potential are lost at the DN2 stage, at which point these cells are committed to the T cell lineage. Expression of the recombinases, RAG1 (Recombinase Activating Gene 1) and RAG2, leads to the rearrangement of the T cell receptor (TCR) γ, δ and β loci at the DN3 stage of development and the diversification into either the αβ or γδ lineage of T cells (16).

Signals emanating from a correctly assembled receptor are required for further survival and differentiation of developing thymocytes. For αβ T cells, this receptor is a complex of CD3, TCRβ and the invariant pre-TCRα chain (pTα). Assembly of a functional TCRβ/pTα complex, a process called beta-selection, leads to αβ lineage commitment, several rounds of proliferation and recombination of the TCRα locus to produce a mature αβ antigen receptor that is expressed on the cell surface. Following this, expression of pTα is lost and upregulation of CD8 and CD4 co-receptors commits precursors to the DP (double positive) stage of thymic development (17). The ligand specificity of the αβ receptor dictates whether that cell will proceed to the CD4 single positive (SP) or CD8SP stage. The first requirement for further selection is the expression of a TCR that can bind to either MHC class I or MHC class II. This process of screening for MHC restriction of T cells is called *positive selection*. While the numbers of αβ TCRs generated by random recombination is enormous, the generation of TCRs that can bind to MHC is quite infrequent, and as a result the majority of DP thymocytes are deleted despite expressing surface TCRs. As most

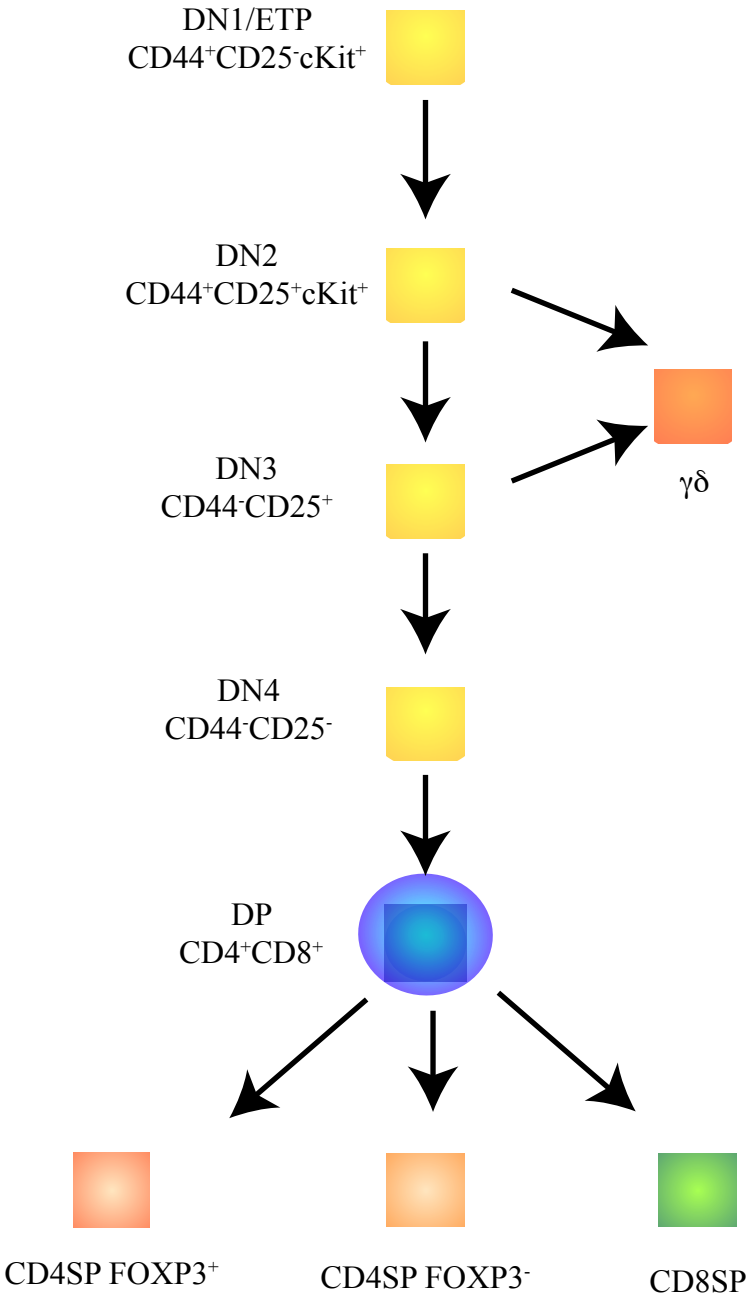

Figure I-1: T cell development in the thymus

Figure I-1 T cell development in the thymus

The earliest precursor cells that seed the thymus are the early thymic precursors or ETPs, which are phenotypically characterized as being $CD4^-CD8^-$ (DN) $CD44^+CD25^-$ and c-Kit^+. These cells then undergo ordered differentiation to become DN2 ($CD44^-CD25^+cKit^+$), DN3 ($CD44^-CD25^+$) and DN4 ($CD44^-CD25^-$) cells. The γδ lineage of T cells arises from DN2 and DN3 precursors, while DN3 cells that express pre-Tα chain are committed to the αβ lineage of T cells. DN4 cells rapidly upregulate expression of CD4 and CD8 to become DP ($CD4^+CD8^+$) cells. DPs that express T cell receptors with appropriate affinity for self-MHC differentiate further to become either CD4SP ($CD4^+CD8^-$) or CD8SP ($CD8^+CD4^-$) cells. DP cells that express the transcription factor FOXP3 mature to become T regulatory cells.

MHC molecules display a self-peptide, thymocytes expressing TCRs that are MHC-restricted also encounter self-antigen in the context of MHC. The quality and quantity of self-antigen that a thymocyte encounters will determine if it will be selected for further development or not. The ligands that induce positive selection of DP thymocytes have lower affinity and avidity to TCRs and are generally not stimulatory for mature T cells in the periphery. On the other hand, MHC-ligand complexes that bind with a high affinity to TCR are detrimental to T cells and signal for apoptosis of T cells. This process, called *negative selection*, is an extremely rapid and sensitive process and is a major mechanism preventing self-reactivity of T cells in the periphery. The DNA-binding transcriptional regulator, AIRE, is one factor that controls this process. AIRE regulates the thymic transcription of genes encoding peripheral tissue antigens that are displayed on medullary epithelial cells and thymic dendritic cells, and impaired presentation of self-peptides in the thymus of *Aire*$^{-/-}$ mice results in fatal autoimmunity in the periphery. The transcription factor ThPOK is essential for DP cells to transit to the MHC class II restricted CD4SP stage, while the transcription factor RUNX3 plays an important role in *Cd4* gene silencing in MHC class I restricted precursors that become CD8SP (18). A small frequency of CD4SP cells is also positive for FOXP3, a transcription factor whose expression is initiated in certain DP precursor cells and maintained in cells that are called regulatory T cells (Treg). CD4SP, CD4SPFOXP3^{+} and CD8SP cells then exit the thymus, homeostatically proliferate and populate peripheral lymphoid organs.

Peripheral tolerance

While intrathymic deletion of self-reactive T cells is an efficient way to ensure T cell self-tolerance, the diversity in antigen receptors and the random recombination events that generate this diversity make the process of deletion in the thymus imperfect. Incomplete presentation of antigens in the thymus and the plasticity of TCR recognition of antigen constitute an inherent danger to the maintenance of tolerance. It is clear that some self-reactive T cells do exit the thymus into the periphery. Additional post-thymic regulatory mechanisms are required to ensure that these auto-reactive T cells are not aberrantly activated in the periphery, and these constitute mechanisms of 'peripheral tolerance'.

Several factors contribute to the maintenance of T cell tolerance to self-antigen in the periphery. Antigen sequestration in immunologically privileged sites such as the eye (19), brain and the testes is one passive mechanism of tolerance by which T cells escape autoimmune activation . The quality and persistence of antigen, the maturation states of both the antigen presenting cell (APC) and the T cell, presence of regulatory cell populations and immuno-modulatory cytokines are some of the other key factors that regulate peripheral T cell tolerance. A description of all these mechanisms of maintaining peripheral tolerance is beyond the scope of this thesis and the subsequent discussions are restricted to mechanisms of dominant suppression utilized by T regulatory (Treg) cells.

Autoimmunity

Failure of the immune system to recognize 'self' and subsequent loss in tolerance results in autoimmunity. This phenomenon, predicted as '*horror*

autotoxicus' by Ehrlich in the late 1800's, is a robust immune response against the host's own tissues originating from inappropriate activation of lymphocytes to self-antigen that results in considerable damage, and often times death, of the host. There are more than 80 described autoimmune diseases that range from systemic diseases, such as SLE (systemic lupus erythematosus), to tissue specific disease, such as Type 1 Diabetes. While every person carries a circulating self-reactive T and B cell repertoire, certain individuals are predisposed to develop autoimmunity. There is a strong linkage between genes of the MHC and polymorphisms in cytokine and costimulatory molecules with autoimmune diseases. In addition, environmental factors like infections also predispose to precipitation of autoimmunity.

Immune Homeostasis

To appreciate the consequences of a breakdown in peripheral tolerance and ensuing autoimmunity, it is important to understand how lymphocytes are sustained at relatively constant numbers in an antigen inexperienced host. Naive lymphocytes in the periphery are maintained as a non-proliferating population with a half-life of at least 6 months (20). Peptide/MHC interactions and cytokine signals are two key factors that regulate the size and composition of the peripheral T cell pool. The common gamma-chain cytokines, IL-7, IL-15 and IL-2 are critical in maintaining peripheral T cell homeostasis. *Il7*$^{-/-}$ and *Il7r*$^{-/-}$ mice have severe defects in lymphocyte development in the thymus as well as maintenance of T cells in the periphery (21). Mice with gene knockouts (KO) of IL-2, IL-2Rα and IL-2/15Rβ all exhibit spontaneous activation of T cells and inflammatory disease, which leads to premature death (22-24). However, unlike *Il7r*$^{-/-}$ mice, the defect in these mice stems from the

lack of an important regulatory cell population that results in abnormal homeostatic regulation of lymphocytes in the periphery (25). In addition to receiving survival signals from cytokine receptors, T cells also constantly interact with MHC-ligand complexes through their T cell receptor. Whether this interaction is essential for T cell survival remains debatable. In experiments with lymphopenic mice, Class II expression appears to be critical for the persistence of adoptively transferred $CD4^{+}$T cells *in vivo* (26, 27). However, in lymphocyte sufficient mice, the same is not true and MHC Class II ligands seem to be dispensable (28-30). Interestingly, tonic self-MHC class II and TCR interactions are essential to ensure *functional* sensitivity of T cells upon encounter with cognate peptide ligands (31).

Peptide ligands are presented to T cells on MHC molecules expressed on antigen presenting cells (APC) such as dendritic cells (DC). The context of T cell and APC interaction determines whether tolerance is maintained or an adaptive immune response is initiated. In a homeostatic environment, DCs are in an immature state and at a given time, about half of the lymph node DCs have high MHC Class II expression and express low levels of co-stimulatory ligands such as B7-2 and ICAM-1 (32, 33). In such steady-state conditions, immature DCs circulate between non-lymphoid and lymphoid tissue at a rapid rate, and have a half-life of <2 days (34-36). Although these DCs constantly scavenge tissues (37, 38), they have poor antigen processing capacities and much of their MHC is loaded with peptides derived from self-tissue (39). In fact, this persistent basal level of presentation of self-peptide is essential for maintaining tolerance, and T cells make short-lived tolerogenic encounters with DCs that result in abortive T cell activation. On the other hand, maturation of DCs results

in increased formation of stable MHC-peptide complexes, higher expression of costimulatory molecules like B7-2, synthesis of cytokines and altered patterns of chemokine and chemokine receptor expression. Under such conditions, DCs can efficiently engage and activate T cells that lead to the initiation of an adaptive immune response. Thus, tolerogenic and immunogenic interactions between T cells and APCs are critical in maintaining the balance between tolerance and immunity.

Maintaining tolerance in the periphery: Regulatory T cells

The immune system has evolved numerous ways to keep autoreactive T cells in check in the periphery. Probably the most prominent pathway of dominant regulation is mediated by the action of suppressor T cells. In the past few years there has been a tremendous growth in our understanding of these cells with respect to their development and function and their potential use as immunotherapeutic agents. It is interesting, however, that immunologists were introduced to this concept as early as 1970 when Richard Gershon reported the presence of a specialized thymic-derived population of suppressor T cells (40). This idea was met with a lot of resistance and was summarily discarded for almost two and a half decades. One of the chief reasons for this skepticism stemmed from the inability of researchers to find the I-J locus, which was thought to encode for the so-called antigenic determinants expressed by suppressor T cells (41). Sadly, Richard Gershon died in 1983, long before the revival of T suppressor cells, and in an obituary by Baruj Benacerraf in the Journal of Immunology, one paragraph stands out- '*Richard Gershon made one of the most important discoveries in Immunology in this century. In 1970 together with his student Kondo he established that* ***thymus-derived lymphocytes are capable of***

exerting a specific negative regulatory effect *in immune responses and called these cells* ***suppressor T cells****….When pioneer scientists endowed with vision make unexpected discoveries, as in the case of Gershon in 1970, they have a difficult time getting their discoveries to be taken seriously..*' (42). In 2002, Charlie Janeway Jr. wrote in the Annual Reviews of Immunology *'(today)...we can infer the existence of the long-anticipated suppressor T cells. So you were right all along, Richard, and I personally apologize for years of my own skepticism*' (43).

An important early observation that influenced the resurrection of dominant suppression in the 1990's was that mice thymectomized during the neonatal stage developed organ-specific autoimmunity and further, cells derived from an adult spleen could prevent this disease (44). Later, Juan Lafaille in Susumu Tonegawa's laboratory demonstrated that TCR transgenic mice specific for the N-acetylated residue 1-11 of the myelin basic protein were completely protected from experimental autoimmune encephalitis (EAE) when injected with the peptide (45). However, if the TCR transgenic mice were crossed onto the RAG deficiency (to eliminate endogenous TCR gene rearrangement), these mice rapidly succumbed to overwhelming EAE upon peptide administration, suggesting the presence of a cell population that was present or induced in RAG sufficient mice that protected from disease. However, the first formal description of a naturally occurring regulatory T cell (Treg) population came from the work of Shimon Sakaguchi who showed that approximately 10% of peripheral $CD4^+$ T cells in an antigen-inexperienced mouse expressed CD25, the IL-2Rα chain. These $CD4^+CD25^+$ T cells behaved as suppressor T cells because transfer of $CD25^+$ depleted $CD4^+$ T cells into *nu/nu* (athymic mice/SCID, discussed later)

mice resulted in severe autoimmunity, which could be prevented by the injection of $CD4^+CD25^+$ cells (46, 47). Interestingly, Fiona Powrie had already noted that transfer of purified naïve T cells lacking expression of activation and/or memory markers into immunodeficient hosts lead to autoimmunity in the form of Inflammatory Bowel Disease (IBD), and similar to Sakaguchi's observation, addition of T cells that express low levels of OX-22, a memory T cell marker, prevents disease (48, 49). Finally, work from Ethan Shevach's laboratory showed that purified $CD4^+CD25^+$ T cells could suppress the proliferation of responder T cells *in vitro*, and also described the IL-2 dependence of these Treg cells for their survival and proliferation *in vitro* (50).

These seminal papers underscored the fact that autoimmune pathology similar to existing human autoimmune diseases can be reproduced in mice by the depletion of a regulatory T cell population and convincingly resurrected the idea of suppressor T cells that exerted dominant regulation to maintain peripheral tolerance. Since their revival, sizeable insights have been made that provide irrefutable evidence towards the existence and indispensability of these Treg cells.

Regulatory T cells

By definition, a regulatory T cell is a cell that a) has the capacity to *modulate* the quality and/or magnitude of an immune response or the lack of it, and b) the absence of such a regulatory cell population should result in pathologic immune dysregulation. However, the term has mostly come to be associated with some form of suppression of immune reactivity, whether it is activation to stimuli (self or

foreign), production of effector cytokines or movement to sites of inflammation and tissue damage. Using these criteria, several subpopulations of Treg cells can be defined including $CD4^+CD25^+$ T cells (51), IL-10 producing Tr1 cells (52), TGFβ producing Th3 cells (53), $CD8^+$ T suppressor cells (54), natural killer T (NKT) cells (55), $CD4^-CD8^-$T cells (56) and γδ T cells (57).

A detailed discussion of the $CD4^+CD25^+$ cells, which are relevant to this thesis, follows in subsequent sections. However, the other Treg cell subsets merit a few words here. The Tr1 cells were first described as a $CD4^+$T cell subset that are distinct from effector Th1 and Th2 cells, and make copious amounts of IL-10 and TGFβ but little or no IL-2 and IL-4 (58). Naïve $CD4^+$T cells can be induced to become Tr1 cells upon antigen priming in the presence of IL-10. $CD8^+$ Tr1-like cells can also be generated *in vitro* by stimulating naïve $CD8^+$T cells with activated plasmacytoid DCs (59) or IL-10 treated myeloid DCs (60). Tr1 cells do not normally express the transcription factor FOXP3 (61), which is a characteristic marker of $CD4^+CD25^+$ Treg cells. However, there are some instances when Tr1 cells do up-regulate FOXP3 upon activation (62). IL-10 and TGFβ primarily mediate the suppressive function of Tr1 cells. These immunomodulatory cytokines act in part by inhibiting IL-2 production by T cells and down-regulating expression of costimulatory molecules and suppressing cytokine production by APCs. An important point to note here is that natural Treg cells ($CD4^+CD25^+FOXP3^+$) also produce IL-10, and the lack of specific Tr1 cells markers makes the distinction between these cells and natural Treg cells difficult.

A regulatory T cell subset important in the induction of oral tolerance and maintenance of homeostasis in mucosal tissues is the TGFβ secreting Th3 cells. Oral tolerance is the specific suppression of cellular and/or humoral responses to an antigen by prior oral administration of the antigen. This is a major pathway of maintaining tolerance to food antigen and commensal bacteria resident in the mucosal surface of the gut. There are two pathways to the induction of tolerance - the generation of regulatory cells and clonal deletion/anergy. Low antigen doses favors the generation of regulatory T cells whereas high dose antigen favors the induction of anergy. Th3 cells, also called 'adaptive' Treg cells, are induced by antigen stimulation in the periphery, as opposed to 'natural' Treg cells that are thymus derived. Th3 cells function by the secretion of immunosuppressive cytokines like TGFβ that can modulate APC function as well as effector T cell differentiation. $CD8^+$ T cells can also be converted to Th3 cells (63), which can mediate bystander suppression *in vivo*.

Besides conventional αβ T cells, non-conventional T cells like the γδ T cells and NKT cells have been shown to have immunoregulatory function. Unlike αβ T cells, most γδ T cells and NKT cells do not undergo negative selection, but are positively selected by engagement of autoantigens during development (64-66). Although the antigen receptor specificity of these cells is unknown, it is clear that the absence of these cell subsets in mice leads to immune dysfunction. γδ T cell-deficient mice present with exaggerated immune responses and exacerbated pathology to infections with *Klebsiella*, *Mycobacteria* and *Listeria* (67-69). The αβ T cell associated lupus-like autoimmunity in MRL/LPR mice is worsened on the γδ-deficient background (70). These studies and others implicate γδ T cells in regulating

aspects of αβ T cell function that can lead to autoimmunity and pathology particularly in intra-epithelial tissues of the gut and skin.

$CD4^+CD25^+$ Treg cells

It is well established that of the numerous subsets of Treg cells, the $CD4^+CD25^+$ Treg cells are the most crucial regulators of peripheral tolerance. The IL-2Rα chain or CD25 was the first molecule to be identified as an important cell-surface marker of Treg cells (46). This is a useful marker to identify Treg cells in an antigen-inexperienced, relatively naïve mouse. However, activated T cells also express CD25, and the utility of CD25 as a marker of Treg cells during an ongoing immune response becomes questionable. Further, CD25 is not simply a marker of activation on Treg cells but is important for Treg cell development and maintenance. Evidence for this comes from the severe autoimmune phenotype of *$Il2^{-/-}$* and *$Il2R\beta^{-/-}$* mice. These strains have a dramatic decrease in the frequency of Treg cells, both in the thymus and periphery. STAT5 (Signal Transducer and Activator of Transcription) is an indispensable signal transducer of common-gamma chain cytokines including IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. While STAT-5 deficient mice (conditional deletion using *Cd4*CreTg mice) have a severe block in thymic development, they also have a significant reduction in the frequency and numbers of $CD4^+CD25^+FOXP3^+$ Treg cells, and it was shown that STAT5 plays an essential regulatory function in *Foxp3* gene transcription downstream of IL-2 (25, 71, 72).

An important development in the field was the identification of FOXP3 (Forkhead Box Protein P3) as a transcription factor that is expressed exclusively in

Treg cells. FOXP3 belongs to the 'forkhead' family of transcription factors that have diverse roles in the immune system.

FOX proteins in immune function

FOX (forkhead) proteins were first identified in *Drosophila melanogaster,* where they are involved in terminal pattern formation in the embryo (73). They are a large family of transcriptional regulators and typically contain a sequence of 80-100 amino acids that form the DNA binding forkhead domain. This motif is also referred to as the winged-helix domain due to the butterfly-like appearance of the loops in the protein structure. There are more than 100 forkhead containing proteins that can be divided into 15 classes based on structural homology (74). FOX proteins play important roles in regulating expression of genes involved in cell growth, proliferation, differentiation, longevity and embryonic development. Several FOX family members regulate different aspects of immune system development and function. Mutations in *Foxn1* are responsible for the nude/SCID (nu) phenotype in mice, rats and humans that results in the lack of hair, abnormal epidermis development and the absence of the thymus (75, 76). Athymia has been attributed to a block in the development of thymic epithelial cells (TEC) in *Foxn1* deficient mice (77). FOXJ1 is a protein that regulates Th1-Th2 effector cell differentiation. *Foxj1* knockout mice are embryonic lethal as FOXJ1 has crucial roles in the development of ciliated cells like pulmonary epithelial cells. However, in fetal-liver chimera experiments, while lymphocyte development is normal, peripheral $CD4^{+}$T cells have an activated phenotype and are prone to producing more IFNγ and IL-2. These cells also have up-regulated expression of *t-bet*, a Th1 skewing transcription factor,

suggesting that FOXJ1 is a negative regulator of Th1 differentiation (78). FOXO3A is a regulator of NF-κB activity and its importance in immune cells came from the evidence that older *Foxo3a*$^{-/-}$ mice suffered from late-onset autoimmunity leading to a dramatic loss in immune homeostasis, lymphoproliferation and multi-organ lymphocyte infiltration (79). One proposed molecular mechanism of FOXO3A function is by regulating the activity of the cell cycle inhibitor, p27kip1 (80), which is a regulator of T cell anergy (81). While these FOX family members regulate significant aspects of immune function, probably the most vital forkhead transcription factor for the immune system is FOXP3.

FOXP3 and Treg cells

The history of FOXP3 can be traced back to 1949, when the *scurfy* mutation arose spontaneously in the inbred MR mouse strain at the Oak Ridge National Laboratory (82). The defect was mapped to a X-linked recessive mutation with lethality in hemizygous males. The affected males (*sf*/Y) suffered from acute autoimmunity leading to ear thickening, scaling of the ears, eyelids, feet and tail. The mice were severely runted and died by three weeks of age because of massive lymphoproliferation. A frameshift mutation in exon 8 of the *Foxp3* gene that generated a protein that lacked the forkhead DNA-binding domain was determined to be the cause of the phenotype of these mice (83, 84). A similar disease is seen in IPEX (Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked) patients who present with watery and often times bloody diarrhea in infancy, and multi-organ autoimmunity including atopic dermatitis, thyroiditis and Type 1 Diabetes (85). Affected individuals usually develop symptoms early in childhood and die by 2-3

years of age. The gene defect responsible for IPEX syndrome was also mapped to mutations in the *Foxp3* gene (84, 86, 87). Heterozygous females carrying a single mutant *scurfy* allele (*sf*/X) were protected from disease despite random inactivation of one X-chromosome, suggesting the existence of a subset of '*Foxp3 wt*' cells that regulate autoimmune activation of lymphocytes in *trans*. These cells that expressed *Foxp3* and could regulate in trans were identified to be the same $CD4^+CD25^+$ Treg cells described by Sakaguchi et al in 1995 (88-90).

A bulk of literature now exists that suggests that FOXP3 is necessary and sufficient for the development and function of Treg cells. The first evidence came from the similar autoimmune phenotype of *Foxp3* knock out mice and *scurfy* mice, both of which are a result of a lack of Treg cells in these mice. There are some indications that FOXP3 may play a role in thymic epithelial cell function (91, 92); however, conditional deletion of *Foxp3* in $CD4^+$ T cells using *Cd4*CreTg mice results in a similar breakdown in tolerance as *Foxp3*$^{-/-}$ mice, ruling out the role of TECs in the observed phenotype (93, 94). Secondly, mixed bone marrow (BM) chimera experiments with *wt* and *Foxp3*$^{-/-}$ BM shows that only *wt* BM can contribute to the $CD4^+CD25^+$ Treg compartment in the thymus and periphery, suggesting that *Foxp3* is necessary for the development of Treg cells (89). Third, the generation of *Foxp3* reporter mice allowed the purification of Treg cells independently of CD25, and functional assays showed that FOXP3 expression is sufficient for *in vitro* suppression while CD25 is dispensable (93, 95). Notably, enforced expression of FOXP3 by retroviral infection of conventional $CD4^+CD25^-$ cells imparts regulatory function to these cells and they can suppress proliferation of uninfected responder cells *in vitro* as

well as regulate various autoimmune diseases (autoimmune gastritis, colitis) in adoptive transfer models *in vivo* (88, 89).

More recently, however, the field has begun to question the role of FOXP3 as the 'master' regulator of Treg development and function. One key observation leading to this is the fact that human $CD4^+$T cells can up-regulate FOXP3 expression during the normal course of their activation (96). This was the first suggestion than $FOXP3^+$ Treg cells can be induced *de novo* upon TCR stimulation. Whether the FOXP3 expression in activated human T cells results in the gain of suppressive function is controversial. It has been suggested that only those cells that can maintain stable, high expression of FOXP3 have the capacity to become suppressor cells. In the mouse system, it has been shown that conventional $CD4^+CD25^-FOXP3^-$ cells can be induced to express FOXP3 upon activation in the presence of TGFβ (95, 97, 98). One characteristic of these *de novo* generated $FOXP3^+$ cells is the instability of FOXP3 expression. 'Natural' thymic derived Treg cells maintain FOXP3 expression and suppressive function and constitute a stable Treg lineage. On the other hand, induced Treg cells in both mice and humans appear to have distinct requirements for maintaining FOXP3 expression, and only those cells that sustain high FOXP3 expression acquire regulatory function (99, 100). This suggests that FOXP3 expression by itself is not indicative of a cell with suppressor activity. In addition to *in vitro* data on induced $FOXP3^+$ cells, the phenotype of GFP-knockin-*Foxp3*-knockout mice suggests that there may be a regulator upstream of FOXP3 that determines Treg lineage fate (101, 102). In this system GFP was used to functionally inactivate the FOXP3 protein and also serve as a reporter of *Foxp3* transcriptional

activity. Interestingly, $GFP^{+}Foxp3^{null}$ cells were phenotypically identical to *wt* Treg cells, were anergic and expressed a number of Treg signature genes, such as *Ctla4*, *Gitr* and *Cd44*. However, these cells were not functional and failed to suppress proliferation of responder cells *in vitro* and *in vivo*. These data suggest that while FOXP3 may be necessary for Treg cell function, it is not the 'master' regulator of the Treg lineage, but may serve to reinforce the Treg phenotype, in part by amplifying its own expression.

Regulation of FOXP3 expression

The specific biochemical and molecular factors that regulate *Foxp3* expression are not known. While there are several known regulators of *Foxp3* expression, these factors also regulate Treg cell survival and proliferation, making it hard to determine the precise regulatory circuits affecting *Foxp3* transcription per se. CD28 costimulation, IL-2 and TGFβ are three important inducers of *Foxp3*. The role of TGFβ in regulating *Foxp3* expression and Treg cell function is discussed in detail in later sections. Mice lacking *Cd28* and *Il2* have a drastically decreased frequency of $FOXP3^{+}$ cells in the thymus and periphery, suggesting a role for these molecules in the development of Treg cells in the thymus (103, 104). However, both CD28 and IL-2 are also required for the survival of Treg cells in the periphery, as well as their proliferation upon TCR stimulation. In addition to costimulation, TCR/MHC interactions are also critical for the development of $FOXP3^{+}$ cells in the thymus. Analysis of *Foxp3*-GFP knockin ($Foxp3^{gfp}$) mice revealed that the majority of mature, $CD24^{lo}$ GFP^{+} cells are CD4SP while a few are DP or CD8SP. The development of $DPGFP^{+}$ and $CD8^{+}GFP^{+}$ cells was severely inhibited in $Foxp3^{gfp}MHCI^{-/-}$ ($b2m^{-/-}$)

mice, and similarly, the development of $CD4^+GFP^+$ cells in *Foxp3*^{gfp}*MHCII*$^{-/-}$ (*Ab*$^{b-/-}$) mice was blocked. No Treg cells developed in *Foxp3*^{gfp}*MHCI*$^{-/-}$*MHCII*$^{-/-}$ (*b2m*$^{-/-}$*Ab*$^{b-/-}$) mice, indicating that TCR/MHC interactions drive Treg cell development (93). In addition, TCR transgenic mice on the *Rag*$^{-/-}$ background do not develop Treg cells while those that express RAG protein and can rearrange endogenous TCR genes do have varying numbers of Treg cells (105). There is a further increase in the Treg cell frequency if the TCR-transgenic mice are crossed with transgenic mice expressing the cognate antigen for the particular TCR (106-109). However, the same is not observed with a TCR having lower affinity for the same antigen. Increasing the quantity of agonist peptide also increases the frequency of Treg cells; however, there is also a simultaneous decrease in the numbers of conventional T cells as a result of their inability to be selected (110). Two insights are gained from these observations. First, Treg cells are most likely selected on high-affinity self-interactions in the thymus. Second, Treg cells are less susceptible to signals leading to death by negative selection. An important interpretation of these studies is that Treg cell development in the thymus is instructive- i.e. the ability of a thymocyte to develop into a Treg cells is determined by the strength of the TCR signal it perceives. However, this does not rule out the existence of a 'precursor' population that is fated to become a Treg cell, and it may well be that a 'master regulator' upstream of FOXP3 directs Treg cell lineage commitment.

A major quandary in the field is the lack of information on the antigen specificities of the TCRs on Treg cells. Several studies have been undertaken to address this issue. One approach has been to clone TCRs that are unique to either $CD4^+CD25^+$ Treg cells or $CD4^+CD25^-$ conventional T cells (Tconv) and express them

by retroviral transduction in RAG-deficient TCR transgenic T cells. Upon transfer of these cells into lymphopenic hosts, T cells that are transduced with the Treg cell specific TCRs appear to expand more, consistent with the notion that Treg cells express TCRs with higher avidity for self-ligands (111). Another method that has yielded some contradicting results is the one used by Pacholczyk et al. They generated a mouse model in which the T cells express a single transgenic TCRβ chain and a variety of TCRα chains derived from an unarranged TCRα minilocus (112). This allowed for some endogenous diversity but also restricted the enormous variety that would be generated from an intact TCRα locus. Analyses of these mice revealed that there is considerable overlap between the TCR repertoire usage of Treg cells and Tconv cells, arguing against the idea that Treg cells are selected on high-affinity interaction. In another report, the same group has shown that, in fact, Treg cells mostly recognize non-self antigens and this recognition as opposed to recognition of self-antigen, is the reason for the wasting disease in an adoptive transfer model of Treg function (113). Based on these experimental evidences, an emerging idea in the field is that developing Treg cells in the thymus are not selected on high-affinity self ligands, but that there are *specific* ligands that select for Treg cells, which do not necessarily have to deliver a strong TCR signal (114).

Little is known about the molecular mechanisms regulating *Foxp3* transcription. The *Foxp3* gene is highly conserved across different mammalian species including human, chimpanzee, mouse, rat and dog and contains 11 exons and three highly conserved non-coding sequences (CNS). The *Foxp3* promoter by itself has weak activity in $FOXP3^{+}$ mouse lymphoblast EL4 cells. A recently identified

evolutionary conserved enhancer region that contains NFAT and SMAD binding sites confers strong activity to the promoter (115). The human basal *Foxp3* promoter contains six NFAT and AP1 binding sites that are important for TCR-mediated regulation of the gene (116). NFAT can directly or indirectly induce *Foxp3* expression. Evidence for this is that human T cells can be induced to express FOXP3 upon TCR stimulation in the absence of TGFβ, an event that can be inhibited by the calcineurine inhibitor, Cyclosporine A (116). The fact that *Stat5a/b* knockout mice have a reduced frequency of $FOXP3^{+}$ cells prompted the investigation of STAT5 regulation of Foxp3 expression. A search for STAT binding sequences revealed tandem consensus STAT-binding motifs in the first intron of the *Foxp3* gene. Consequently, it was shown that activated STAT5 and STAT3 (downstream of IL-2R signaling) could bind to these GAS (interferon-gamma activated sequence) motifs (71, 72). In addition to the NFAT, AP-1 and STAT binding sites, the CNS region also contains a putative CREB-ATF (cyclic-AMP response element binding protein/activating transcription factor) binding motif. As CpG methylation inhibits CREB binding, the identification of this motif led to the speculation that *Foxp3* expression may be regulated by DNA methylation. Indeed, a decrease in *Dnmt1* (DNA methyltransferase) expression by RNA-interference (RNAi) in CTLL-2 cells led to an increase in *Foxp3* mRNA and protein expression (117). Similarly, *in vitro* treatment of naive TCR transgenic $CD4^{+}CD25^{-}$ cells with a DNA hypomethylating agent, Azacytidine (Aza), induced *de novo* expression of *Foxp3*, even in the absence of TGFβ (118). Consistent with this, one group identified another CNS upstream of exon-1 that possessed transcriptional activity and was also completely demethylated at all CpG motifs. This region, called the Treg-specific demethylated region (TSDR),

is associated with modified histones and is suggestive of epigenetic imprinting of the *Foxp3* locus (119). Further, while stable *Foxp3* expression in natural Treg cells has been linked to the demethylation of the CNS (120), TGFβ induced Treg cells, which are notorious for their instability in maintaining FOXP3 expression, were shown to harbor intermediate/partial methylation of the CpG motifs within the *Foxp3* locus (119). Thus, while methylation may regulate expression of FOXP3, it remains to be determined if chromatin remodeling at the *Foxp3* locus and expression of *Dnmt1* are necessary for the epigenetic modifications that could provide a basis for Treg lineage determination.

While information on the transcriptional regulation of *Fopx3* expression is far from complete, much more is known about FOXP3 mediated regulation of target genes. It is clear that FOXP3 can act both as a transcriptional activator and a repressor (82) and can regulate the expression of a large number of genes. Co-stimulatory signals from CD28 leads to the activation of AP1 and subsequent binding with NFAT (activated by TCR signals) which cooperatively initiates the transcription of the *Il2* gene. FOXP3 cooperatively binds to NFAT (121) and is involved in a regulatory circuit involving a transcriptional switch, whereby NFAT-AP1 binding to the *Il2* gene is replaced by NFAT-FOXP3 binding. FOXP and AP1 occupy the same DNA region on the *Il2* promoter but interact with different residues on NFAT. Further, NFAT-AP1 complexes activate, while NFAT-FOXP3 complexes repress *Il2* expression. Conversely, NFAT-FOXP3 complex binding to the promoter regions of *Ctla4* and *Cd25* acts as a transcriptional activator, and binding of this complex to these sites is enhanced by TCR stimulation, consistent with the idea that Treg cells need to be

activated through their TCR to initiate their regulatory program. Another transcriptional partner of FOXP3 is RUNX1. The *Il2* promoter contains three RUNX consensus sites and both RUNX1 and RUNX3 can bind and positively regulate *Il2* gene transcription (122, 123). Interestingly, RUNX1 and RUNX3 can also bind to FOXP3 and act as transcriptional repressor of the *Il2* gene. It has been suggested that FOXP3-NFAT-RUNX1 can form a higher order complex and cooperatively regulate gene transcription. This would be possible because while NFAT and FOXP3 interact by direct contact between their DNA-binding domain, FOXP3 and RUNX1 interact through regions distinct from their DNA-binding domains on sites that are widely separated on the genome (121). The N-terminal domain of FOXP3 is unique in that it lacks the CtBP (transcriptional repressor) binding motif that is present in all other FOXP family members (124).

In the search for FOXP3 regulated genes, two groups reported that FOXP3 could bind to approximately 1200 genomic regions (125, 126). FOXP3 regulated genes are primarily modulators of T cell activation and consist of transcriptional regulators, intracellular signaling and cell surface receptors. The promoters of genes expressed in Treg cells, such as *Il2*, *Cd25*, *Gitr*, *Ctla4*, *Il7ra*, *Icos, Pde3b* and *Ccr4,* are bound by and regulated by FOXP3. Interestingly, direct FOXP3 target genes comprise only 6% of the entire program of FOXP3 dependent gene expression, suggesting that FOXP3 may regulate other transcription factors that regulate further transcriptional regulation. A few such unique transcription factors that are expressed in both the thymic and peripheral Treg cells are *Prdm1*, *Crem*, *Zfn1a2* and *Irf6*; and transcription factors that are differentially expressed only in thymic Treg cells are

Stat5, *Irf4*, *Hif1a* and *Stat4*. In addition, FOXP3 regulates expression of a few noncoding RNA sequences, of which *mir155* is the most prominent.

Mechanisms of function of Treg cells

FOXP3^{+}Treg cells can be divided into two subsets- natural Treg cells (nTreg) that develop in the thymus and induced Treg (iTreg) cells that are generated from conventional cells in the periphery. Both these subsets utilize multiple mechanisms to exert immune regulation (**Figure I-2**). However, these pathways of regulation are highly context dependent and different experimental readouts of Treg function reveal different mechanisms utilized by Treg cells. The commonly used method to test Treg cell function is the *in vitro* suppression assay, in which TCR-stimulated Treg cells are co-cultured with responder cells (naïve CD4^{+}CD25^{-} T cells or splenocytes) in the presence of irradiated APCs. The ability of Treg cells to suppress the proliferation of responder cells upon their activation is used as a measure of Treg cell activity. In addition, another popular *in vivo* measure of Treg function is the ability of Treg cells to prevent colitis that is induced in mice by the transfer of Treg depleted, naïve CD4^{+}CD25^{-}CD45RB^{hi} conventional T cells. Both these assays are extremely artificial in that neither represents any physiologically relevant scenario where Treg cells might really be important. However, much of the mechanistic insights into Treg function have utilized these assays and they are discussed below.

Immunomodulatory cytokines

Treg cells can function by the production of immunosuppressive cytokines like IL-10 and TGFβ. The role of TGFβ in Treg function is discussed in detail later.

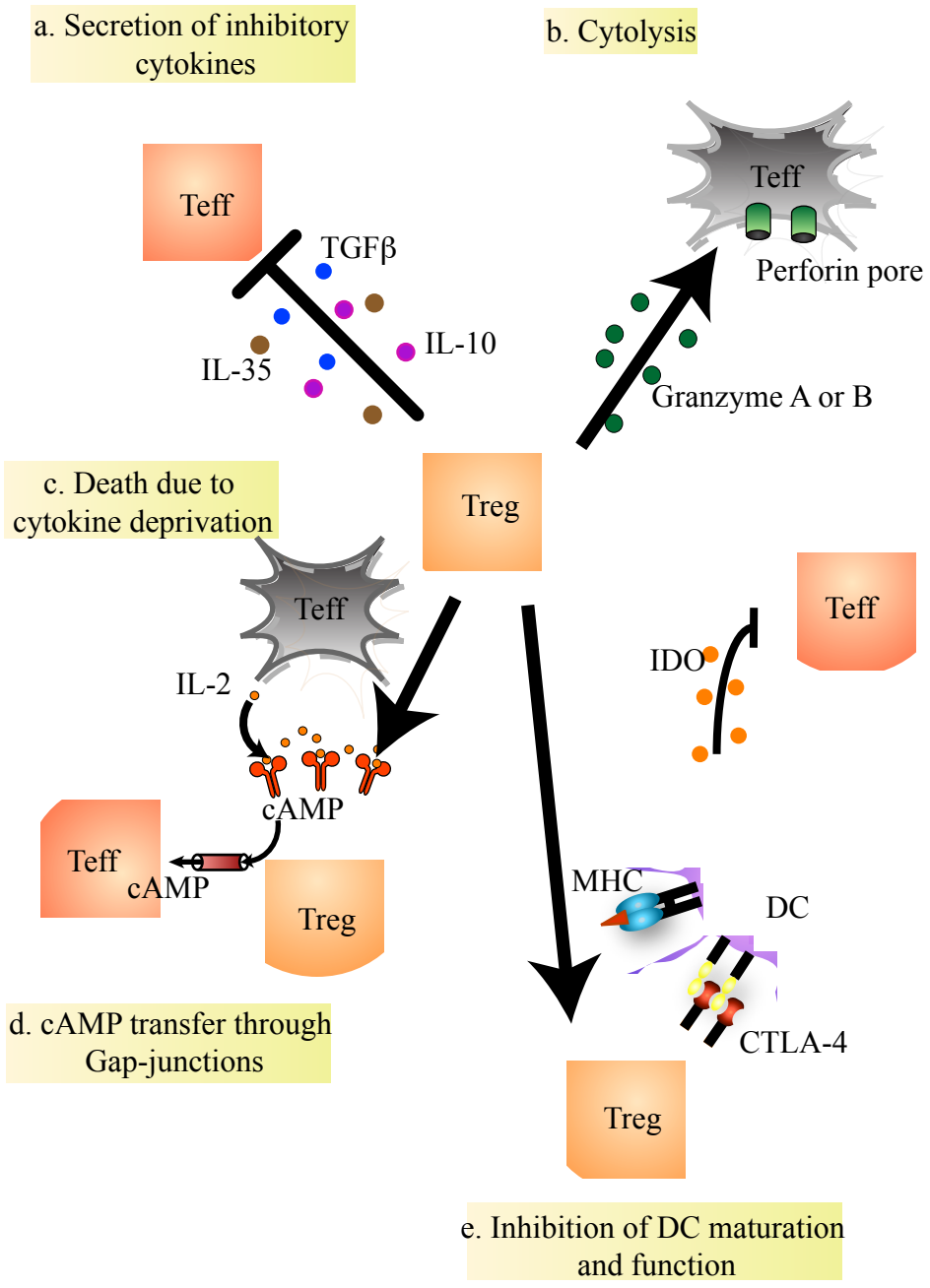

Figure I-2: Mechanisms of Treg cell function

Figure I-2 Effectors of Treg cell function

$FOXP3^+$ Treg cells utilize multiple context dependent mechanisms to regulate immune responses. a) Treg cells can secrete immunosuppressive cytokines such as IL-10, TGFβ and IL-35 that inhibit various aspects of T cell function as well as those of innate cells and antigen presenting cells. b) Treg cells can directly lyse target cells by a perforin dependent mechanism and also by secretion of Granzyme A or B. Other mechanisms of suppression by Treg cells include c) direct cell-to-cell transfer of cAMP via GAP junctions that can inhibit activated T cells by blocking IL-2 production and d) by acting as a 'sink' for the T cell cytokine, IL-2, by virtue of their high expression of CD25. e) Treg cells can also modify antigen presenting cells (APCs) such that they are rendered ineffective in activating T cells.

While FOXP3^{-}CD4^{+} T cells (Tr1 and Th3 cells) also produce IL-10 and TGFβ, natural Treg cells arising in the thymus have been shown in different circumstances to depend on these cytokines for their function (127). IL-10 and TGFβ are not required for *in vitro* suppression by Treg cells, as the addition of neutralizing antibodies to IL-10 and TGFβ (128, 129) or the use of Treg cells from *Il10*$^{-/-}$ T cells (50) does not affect suppression in co-culture assays *in vitro*. However, the role of IL-10 in mediating some aspects of Treg function *in vivo* is indisputable. The clearest experimental evidence comes from the conditional deletion of *Il10* in FOXP3^{+} cells using CRE transgenic mice in which the *Foxp3* promoter drives *Cre*Tg expression in Treg cells (130). In these mice, Treg cell produced IL-10 was not required for the control of systemic autoimmunity but was required to maintain immune homeostasis at sites of environmental interface. The *Il10*$^{flox/flox}$*Foxp3*Cre mice displayed a specific loss of tolerance at mucosal surfaces with severe inflammation and immune pathology of the colon and the lungs. These findings agree with the earlier studies showing that IL-10 (and TGFβ) is necessary to control airway allergic responses by Treg cells (131). IL-10 is also important for the prevention of colitis in mouse models of IBD (inflammatory bowel disease) (132). In addition to controlling autoimmunity, Treg cell derived IL-10 is also important for the control of various infections such as *Mycobaterium* (133) and *Leishmania* (134).

In addition to IL-10, a new inhibitory cytokine called IL-35 that is formed by the pairing of IL-12α (p35) with the Epstein Barr virus induced gene-3 (Ebi3), is also important for Treg cell function. Expression of IL-35 is increased in Treg cells after activation and is required for maximal suppression by Treg cells (135). While *Ebi3*$^{-/-}$

mice do not suffer from overt autoimmunity, Treg cells from *Ebi3*$^{-/-}$ mice function very poorly to control autoreactive T cells in a colitis model and a homeostatic proliferation model of Treg function. The mechanism of IL-35 function remains to be determined.

Cytotoxicity of Treg cells

Although cytotoxic killing is primarily associated with the activity of NKT cells and CD8^{+} T cells, human and mouse Treg cells have been shown to up-regulate expression of Granzyme A and B respectively, and perforin after activation (136, 137). Granzyme B deficient mice can clear tumor cells more efficiently than *wt* mice and transfer of *wt* Treg cells into Granzyme B deficient mice restores their susceptibility to tumor growth. This suggests that one mechanism by which Treg cells could maintain a suppressive tumor microenvironment was by Granzyme B mediated killing of effector T cells (138). Indeed, Treg cells from Granzyme B-deficient mice have compromised suppressive activity *in vitro* (138, 139). A role for Granzyme B in Treg mediated function is further supported by the fact that over-expression of a Granzyme B inhibitor in responder T cells makes them immune to suppression in vitro (140).

Cytokine deprivation and disruption of metabolic function

Treg cells constitutively express high levels of the IL-2Rα chain, CD25, on their surface. Because of this, Treg cells have been proposed to serve as an IL-2 'sink', depriving other responding T cells in the vicinity of IL-2 and thus limiting their proliferation (50, 141) and inducing apoptosis (142). Cyclic adenosine

monophosphate (cAMP) is an important second messenger that is a potent inhibitor of T cell proliferation and differentiation (143). It was recently shown that Treg cells could directly transfer cAMP into effector T cells though membrane GAP junctions (144). Expression of the ectoenzymes, CD39 and CD73 generates pericellular adenosine by Treg cells, which suppresses effector T cell function by the activation of the adenosine receptor 2A ($A_{2A}R$) (140).

Suppression of DCs

In addition to having a direct effect on effector T cells, Treg cells can also modulate dendritic cell function, which can then affect function of responding T cells. Intravital studies have revealed that Treg cells directly interact with DCs *in vivo* (145, 146). Further, upon TCR stimulation, highly motile Treg cells preferentially aggregate around DCs in an LFA-1 dependent manner. Upon aggregation, Treg cells specifically down-regulate the expression of B7-1/86 but not CD40 and MHC class II on DCs (147). Much of the modulation of DC function has been linked to CTLA-4 expression on Treg cells, and the essential role of CTLA-4 in addition to DC regulation in Treg function is discussed in detail below.

Intensive investigations are currently in progress to understand the complexities of Treg function. The original idea that Treg cells function by cell-contact dependent mechanisms clearly needs to be revised. Further, as more insight is gained into the Treg cell functional arsenal, the redundancy in Treg cell function is becoming more apparent. This is demonstrated by the fact that none of the molecules/pathways described above result in a complete lack in regulatory activity

when blocked or disrupted, the consequences of which would lead to a scurfy-like phenotype. However, there are two mouse models of gene deletion that closely resemble the phenotype of *Foxp3*$^{-/-}$ and *scurfy* mice. Similar to *Foxp3*$^{-/-}$ mice, both *Ctla4*$^{-/-}$ and *TGFβ*$^{-/-}$ mice suffer from early onset autoimmunity, leading to multi-organ lymphocyte tissue infiltration and death by 3-4 weeks of age. While both mouse models do generate FOXP3^{+} cells, whether these cells are functional or are the cause of dysregulated immunity in these mice remains unknown. A literature review of these two molecules and their relationship with Treg cells follows in the subsequent sections.

CTLA-4 and peripheral tolerance

Peripheral T cells express TCRs with affinity for self-peptides as a result of positive selection in the thymus. The requirement for two signals for optimal T cell activation has been shown to be critical for the maintenance of peripheral T cell tolerance. The balance between positive and negative signals from different costimulatory ligands determines the ultimate outcome of T cell encounter with antigen to generate an immune response or prevent autoimmunity.

Two-signal model of T cell activation

One important mechanism of maintaining the fidelity of immune responses and avoiding aberrant self-reactivity is the requirement of two signals for T cell activation. In 1959, Lederberg first proposed the idea of two qualitatively different signals perceived by a lymphocyte that resulted in either tolerance or activation (148). He postulated a temporal model of lymphocyte signaling in which TCR binding to

cognate antigen was identical in immature and mature cells, but the signal transduction pathway differed with the state of maturity resulting in two biologically different outcomes. Bretscher and Cohn modified this theory to explain B cell tolerance by suggesting that lymphocyte activation required 2 signals (148). Signal one was the occupancy of the antigen-specific receptor, which by itself led to inactivation of B cells. If however, the B cell received a second signal in conjunction with the antigen receptor signal, this would lead to its activation and antibody production. They proposed that the second signal in their model came from T cells that provided 'help' to B cells.

To explain why certain stimuli induced a state of unresponsiveness in T cells while others activated them, Jenkins and Schwartz refined the original 2-signal model to apply to T cell activation. They showed that peptide-MHC complexes (Class II Ia) on planar lipid membranes alone in the absence of accessory cells failed to activate T cells and induced a state of proliferative unresponsiveness in them (149). Similarly chemically modified splenocytes also induced antigen-specific unresponsiveness *in vitro* and *in vivo* (150). Today, an enormous body of literature exists which clearly demonstrates that optimal T cell activation requires an antigen specific signal from the TCR that interacts with MHC-peptide complexes on APCs, and a second, antigen independent signal called the co-stimulatory signal also provided by mature APCs. TCR signals alone in the absence of co-stimulation leads to the induction of anergy in naïve T cells.

CD28 costimulation

A prominent costimulatory molecule for naïve T cells is CD28 (151-153), which is constitutively expressed on most circulating T cells in mice and humans. Although additional costimulatory pathways exist, including CD40/CD40L, Ox40/Ox40L, and LFA-1/ICAM-1, the CD28 pathway is the most potent and well studied amongst them. The ligands for CD28 are B7-1 (CD80) and B7-2 (CD86), which are upregulated on APCs during their differentiation induced by various stimuli, and are also upregulated on T cells after activation (154). CD28 binds to these molecules with relatively low affinity and very fast association and dissociation rate constants (155). While CD28 binds to B7-1 with approximately 10-fold higher affinity than B7-2, B7-2 is the preferred ligand for CD28 *in vivo*. Under steady-state conditions, CD28 mediates cell-cell adhesion and facilitates conjugate formation and T cell interaction with APC. Because of the relative high density of CD28 expression and the rapid dynamic nature of its interaction with its ligands on APCs, CD28 on T cells helps TCRs scan APCs for rare ligands and plays a role in the initiation of T cell responses.

CD28 binding to its ligands, B7-1 and B7-2, initiates a signaling cascade, which results in increased IL-2 receptor expression, IL-2 transcription and mRNA stabilization, induction of anti-apoptotic proteins like Bcl-xL (156) and enhanced glucose metabolism (157) in T cells. In addition, CD28 signals decrease the threshold of T cell activation by the formation of stable immunological synapses. CD28 signals by themselves cannot induce T cell activation and proliferation, and it is generally thought that CD28 functions primarily to potentiate TCR signals.

Cytotoxic T lymphocyte Antigen-4 (CTLA-4)

Ctla4 was identified by differential screening of a murine CTL (cytotoxic T lyphocyte) cDNA library as a gene involved in the function of CTLs (158). It encodes for a 2kB transcript that translates a 223 amino acid protein whose expression is restricted to lymphocytes. It was quickly established that CTLA-4 was closely related to CD28 in terms of sequence, message expression, gene structure and chromosomal location and it was hypothesized that CTLA-4 arose as a result of a gene duplication event (159). Indeed, the *Ctla4* and *Cd28* genes are separated by only 25-150 kilobases (160). Further, a soluble version of the CTLA-4 ectodomain fused to an immunoglobulin tail (CTLA-4-Ig) bound to B7-1 and B7-2 on APCs similar to CD28 but with considerably higher affinity (161). It was also shown that CTLA-4-Ig worked as an immunosuppressive agent *in vivo* (162) and promoted donor-specific tolerance and survival of xenogeneic islet grafts (163) by preventing T cell activation.

The first hints at the function of CTLA-4 in regulating T cell responses came with the generation of CTLA-4 monoclonal Abs. However, while the anti-CTLA-4 mAb efficiently bound to CTLA-4, it was unclear if the antibody blocked CTLA-4 signals or cross-linked the receptor to transmit a CTLA-4 signal. As a result, the earliest studies misinterpreted the increased proliferation observed upon the addition of anti-CTLA-4 Ab in conjunction with anti-TCR and anti-CD28 Abs, and concluded that '*CTLA-4 (with CD28) cooperatively regulates T cell adhesion and activation by B7*' (164). The first suggestion that CTLA-4 may be an inhibitory receptor on T cells came from the studies comparing the effects of intact and monovalent fragments of

the CTLA-4 mAB. While both intact and monovalent fragments of mouse anti-CTLA-4 mAb enhanced T cell responses in allogeneic MLR reactions, only the intact Ab inhibited proliferation under conditions where Fc receptor cross-linking was provided (165). This was confirmed in experiments where cross-linking of the anti-CTLA-4 mAb was achieved using a secondary anti-hamster Ab (166). Finally, *in vivo* studies revealed that anti-CD28 blocking antibodies inhibited whereas anti-CTLA-4 blocking antibodies enhanced T cell responses (167, 168), suggesting that CD28 and CTLA-4 have opposing effects on T cell activation.

Ctla4$^{-/-}$ *mice*

The phenotype of *Ctla4* knockout mice is the most conclusive demonstration of the inhibitory function of CTLA-4. *Ctla4*$^{-/-}$ mice develop a severe lymphoproliferative disorder and die by 3-4 weeks of age (169, 170). The majority of data suggest that CTLA-4 deficiency does not affect thymic development (170, 171), although there is one report that suggests that negative selection may be altered (172). The disease in *Ctla4*$^{-/-}$ mice appears to result primarily from a defect in peripheral T cell tolerance and homeostasis (169, 171, 173). Although both CD4 and CD8 T cells are affected, aberrant CD4^{+} T cell activation that begins early at 5 days after birth is considered to be the initiating factor for the lymphoproliferative disease. Massive tissue destruction with severe myocarditis and pancreatitis, lymphadenopathy and splenomegaly ultimately result in death of these mice. *Ctla4*$^{-/-}$ mice expressing a TCR transgene on a RAG-deficient background are protected from disease (170, 174) suggesting that T cells become activated to self-antigen in the periphery of *Ctla4*$^{-/-}$ mice. In addition, B7-CD28 interactions are necessary for the development of disease

in *Ctla4*$^{-/-}$ mice since T cells from *Ctla4*$^{-/-}$*Cd28*$^{-/-}$ mice and *Ctla4*$^{-/-}$*B7*$^{-/-}$ mice are naïve and the mice are healthy (175). Blocking B7 costimulation by CTLA4Ig treatment of neonatal *Ctla4*$^{-/-}$ mice also prevents the development of disease (175, 176).

CTLA-4 protein: isoforms and expression

The *Ctla4* gene consists of four exons: Exon 1 encodes the leader peptide sequence, exon 2 contains the ligand-binding site, exon 3 encodes the transmembrane region and exon 4 codes for the cytoplasmic tail (177, 178). Human and mouse CTLA-4 undergo differential alternative splicing to generate CTLA-4 isoforms (**Figure I-3A**). Humans express the full-length mRNA containing exons 1-4, a transcript encoding a soluble form of CTLA-4 that lacks the trans-membrane domain encoding exon 3, and a transcript encoding only exons 1 and 4. Mouse T cells can express an additional transcript, the ligand-independent isoform that lacks the B7 binding domain encoded by exon 2 (158, 177-182). The expression of different isoforms of CTLA-4 has been associated with susceptibility to autoimmune diseases in humans and mice.

An understanding of the precise transcriptional regulation of CTLA-4 expression is lacking. NFAT is one regulator of CTLA-4 transcription as modulation of NFAT levels by Cyclosporine A correlates directly with CTLA-4 expression (183, 184). There are several additional transcriptional regulatory elements in the 5' UTR of the *Ctla4* gene, including sites for AP-1, STAT, GATA-1, NF-κB and Oct-1 binding (178, 184, 185). cAMP also upregulates CTLA-4 expression and may be involved in directly regulating *Ctla4* transcription (186).

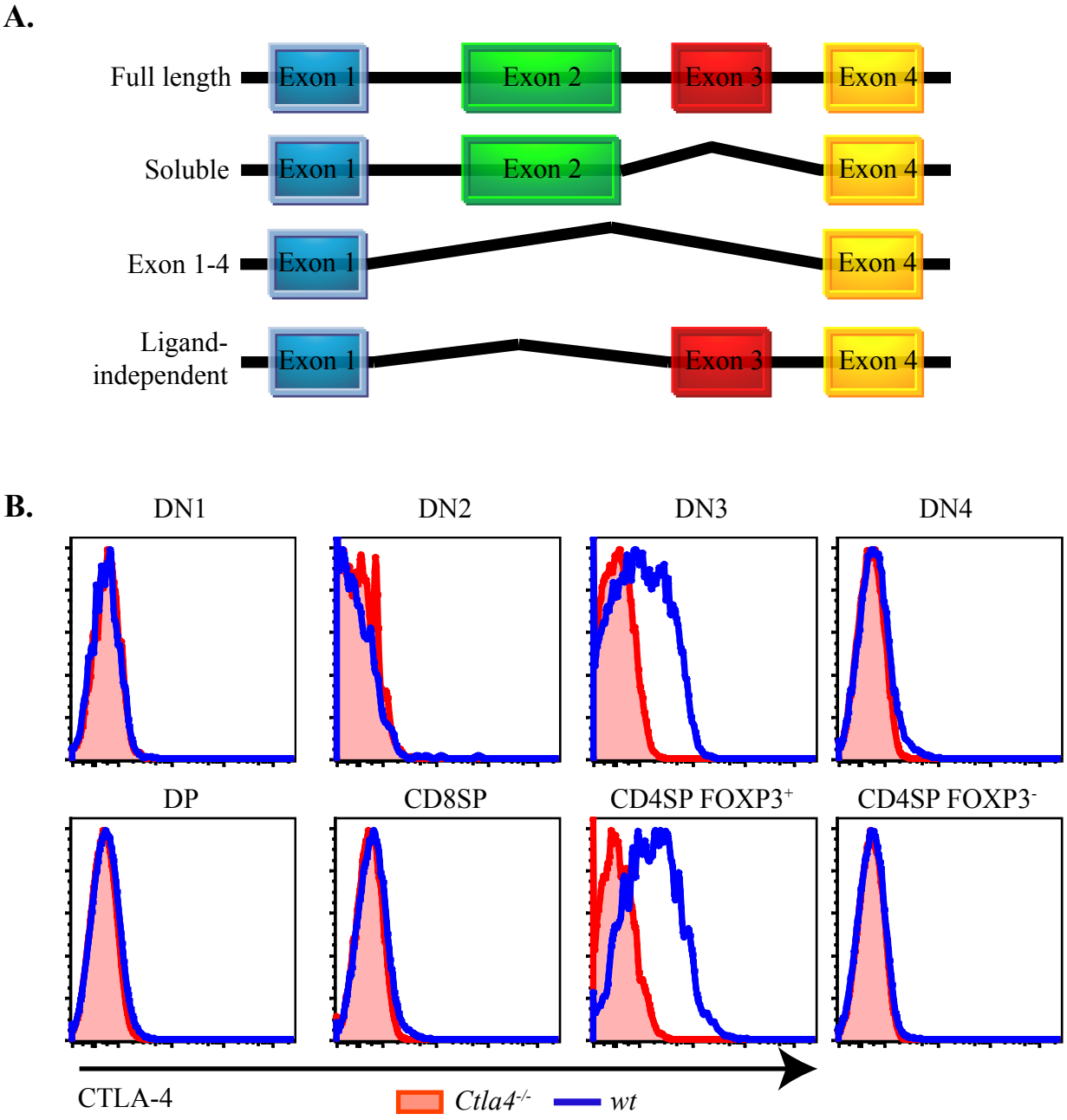

Figure I-3: CTLA-4 isoforms and expression pattern in thymocytes

Figure I-3 CTLA-4 isoforms and expression pattern in thymocytes

A. The *Ctla4* gene consists of four exons: Exon 1 encodes the leader peptide sequence, exon 2 contains the ligand-binding site, exon 3 encodes the transmembrane region and exon 4 codes for the cytoplasmic tail. Alternate splicing of mRNA results in the generation of four isoforms of CTLA-4: full-length mRNA containing exons 1-4, a soluble form of CTLA-4 that lacks the trans-membrane domain encoding exon 3, a transcript encoding only exons 1 and 4 and a ligand-independent isoform that lacks the B7 binding domain encoded by exon 2. The ligand-indepndent isoform is only detected in mice and not in humans.

B. Flow cytometric analysis of CTLA-4 expression in T cell subsets was performed on thymocytes from 3-4 weeks old C57/BL6 mice. Intracellular CTLA-4 was expressed in early precursors cells at the DN3 stage of thymocyte development. CTLA-4 expression was lost in developing αβ T cells and was re-expressed only in Treg cells that were $CD4^{+}FOXP3^{+}$. CD8SP and $CD4SPFOXP3^{-}$ cells did not express detectable intra-cellular CTLA-4. (DN1: $CD4^{-}CD8^{-}CD44^{+}CD25^{-}$, DN2: $CD4^{-}CD8^{-}CD44^{+}CD25^{+}$, DN3: $CD4^{-}CD8^{-}CD44^{-}CD25^{+}$, DN4: $CD4^{-}CD8^{-}CD44^{-}CD25^{-}$, DP: $CD4^{+}CD8^{+}$, CD8SP: $CD4^{-}CD8^{+}$, CD4SP: $CD4^{+}CD8^{-}$)

Despite the significant structural homology, CD28 and CTLA-4 proteins have very different lifestyles. While CD28 is constitutively expressed on T cells, with little change in expression even upon activation (187), CTLA-4 expression is differentially regulated in naïve and activated T cells. Further, the expression pattern of CTLA-4 isoforms also varies between cell subsets. Full-length (fl) CTLA-4 expression can be first detected in early DN3 thymic precursors cells (158, 188) (Jain N unpublished observations, **Figure I-3B**). DP cells do not express detectable flCTLA-4 protein and CTLA-4 is re-expressed only in CD4SP cells that are $FOXP3^{+}$. Activated thymocytes can also upregulate CTLA-4 mRNA expression (189). Treg cell restricted expression of CTLA-4 is also seen in peripheral lymphoid organs (159, 185, 190, 191). Naïve T cells (both $CD4^{+}$ and $CD8^{+}$) do not express CTLA-4 mRNA and protein. However, upon TCR stimulation, CTLA-4 mRNA is rapidly induced within 1 hour (159, 185, 190), and CTLA-4 protein can be detected by 24 hours after activation, with peak expression at 36-48 hours. CD28 co-stimulation and IL-2R signaling are necessary for CTLA-4 mRNA stabilization and protein expression (184, 192). The half-life of CTLA-4 mRNA is 4.6 hours under conditions of TCR stimulation alone, which increases to 8.9 hours with CD28 co-stimulation. Previously activated/memory T cells have a relatively large pool of intracellular CTLA-4, which is rapidly cycled to the cell surface upon activation (193). Compared to naïve T cells, memory T cells can sustain surface CTLA-4 expression for a longer time. The ligand-independent (li) CTLA-4 isoform is expressed at high levels in resting/memory T cells and expression is rapidly downregulated upon activation (180). Besides T cells, CTLA-4 gene expression can be detected in various other cell types such as B cells (194),

granulocytes, $CD34^+$ stem cells, placental fibroblasts (195), and mouse embryonic stem cells (196). However, the significance and functional consequences of CTLA-4 expression in these cells is unknown.

The bulk of CTLA-4 protein remains in intracellular vesicles that localize close to the microtubule organizing center (MTOC) (192, 197). Surface CTLA-4 expression is highly dynamic and tightly regulated by endocytosis. The cytoplasmic tail of CTLA-4 contains a tyrosine-based intracellular localization motif that results in rapid endocytosis of CTLA-4 protein from the surface to endosomal compartments as well as targeting of some CTLA-4 to lysosomes for degradation. The half-life of CTLA-4 protein is only about 2 hours in activated T cells (192, 198, 199). The clathrin-coated pit adaptor protein, AP-2, plays a role in this endocytosis process. CTLA-4 expression on the surface is stabilized by tyrosine phosphorylation (by LCK and ZAP-70 dependent mechanisms) of the endocytosis motif that inhibits AP-2 binding. The cytoplasmic domain of CTLA-4 protein is 100% conserved across species and endocytosis and control of surface CTLA-4 expression may be an important aspect of biological effects mediated by CTLA-4 (200). No functions have been attributed to intracellular flCTLA-4 as yet.

CTLA-4 is expressed as a homodimer on the cell surface (200). The crystal structure of CTLA-4 bound to B7-1 suggests that the bivalent binding of CTLA-4 to B7-1 dimers leads to the formation of lattice structure of alternating CTLA-4/B7 molecules (201, 202). Upon T cell-APC interaction, CTLA-4 accumulates at the immunological synapse (IS) via lipid rafts (203). The amount of CTLA-4 in the IS is

directly proportional to the strength of the TCR signal (204) and CTLA-4 interaction with B7-1 (205).

CTLA-4 polymorphisms and disease susceptibility

As discussed previously, CTLA-4 expression in different T cell subsets is tightly regulated at the transcriptional, translational and post-translational levels. Thus, even subtle alterations in expression of CTLA-4 may have serious immunological consequences. Four main polymorphisms of the *Ctla4* gene have been identified that have repercussions on CTLA-4 expression and subsequent disease susceptibility. Single nucleotide polymorphisms have been noted in human CTLA-4 at positions -1722, -1661 and -318 in the regulatory/promoter region of the gene, and one at position +49 in exon 1. The T-1772C and A-1661G polymorphisms are not well characterized. The A49G polymorphism is the only polymorphism that changes the primary amino acid sequence of CTLA-4, causing an alanine to threonine substitution (200). This mutant form of CTLA-4 is aberrantly processed in the endoplasmic reticulum, leading to decreased surface expression (206). In contrast, the C-138T polymorphism has been associated with increased promoter activity that results in increased CTLA-4 expression (207). An additional dinucleotide (AT) repeat polymorphism in the 3'UTR of the *Ctla4* gene has been identified. Patients with longer AT repeats have T cells with higher proliferative potential upon TCR and CD28 stimulation (208). There are several reports correlating *Ctla4* polymorphisms with susceptibility to autoimmune diseases such as autoimmune thyroid disease including Graves' disease and Hashimoto's thyroiditis, myasthenia gravis, multiple scelorsis and systemic lupus erythematosus (209). However, the data are contentious

and the precise mechanisms by which these polymorphisms affect CTLA-4 expression are unclear.

The role of CTLA-4 in Type 1 diabetes (T1D) is better understood. Short-term therapy with anti-CTLA4 mAbs of a diabetogenic TCR transgenic mouse strain results in insulitis and β-cell destruction (210). The genetic association between T1D and CTLA-4 in humans and mice (NOD) has been well established. CTLA-4 (identified as disease susceptibility locus Idd5.1) is the only costimulatory molecule directly linked to T1D at the genetic level in both species. A polymorphism at position +77 in exon 2 of the *Ctla4* gene that results in the expression of the liCTLA-4 isoform has been detected in mice and is linked to diabetes susceptibility (179, 180). In diabetes resistant mice, base 77 is an adenine that allows for alternative splicing and generation of the liCTLA4 isoform. In diabetes susceptible mice, the adenine is replaced with a guanine that favors the inclusion of exon 2 in the final transcript. This liCTLA-4 is expressed at lower levels in NOD T cells and can inhibit T cell activation *in vitro*. Human CTLA-4 contains a threonine at base 77 instead of adenine, which is probably why humans do not express the liCTLA-4 isoform.

CTLA-4 mechanisms of action

Numerous mechanisms have been proposed for CTLA-4 function and it is quite likely that CTLA-4 utilizes multiple mechanisms operating at different levels to regulate peripheral T cell tolerance (**Figure I-4)**.

Ligand competition

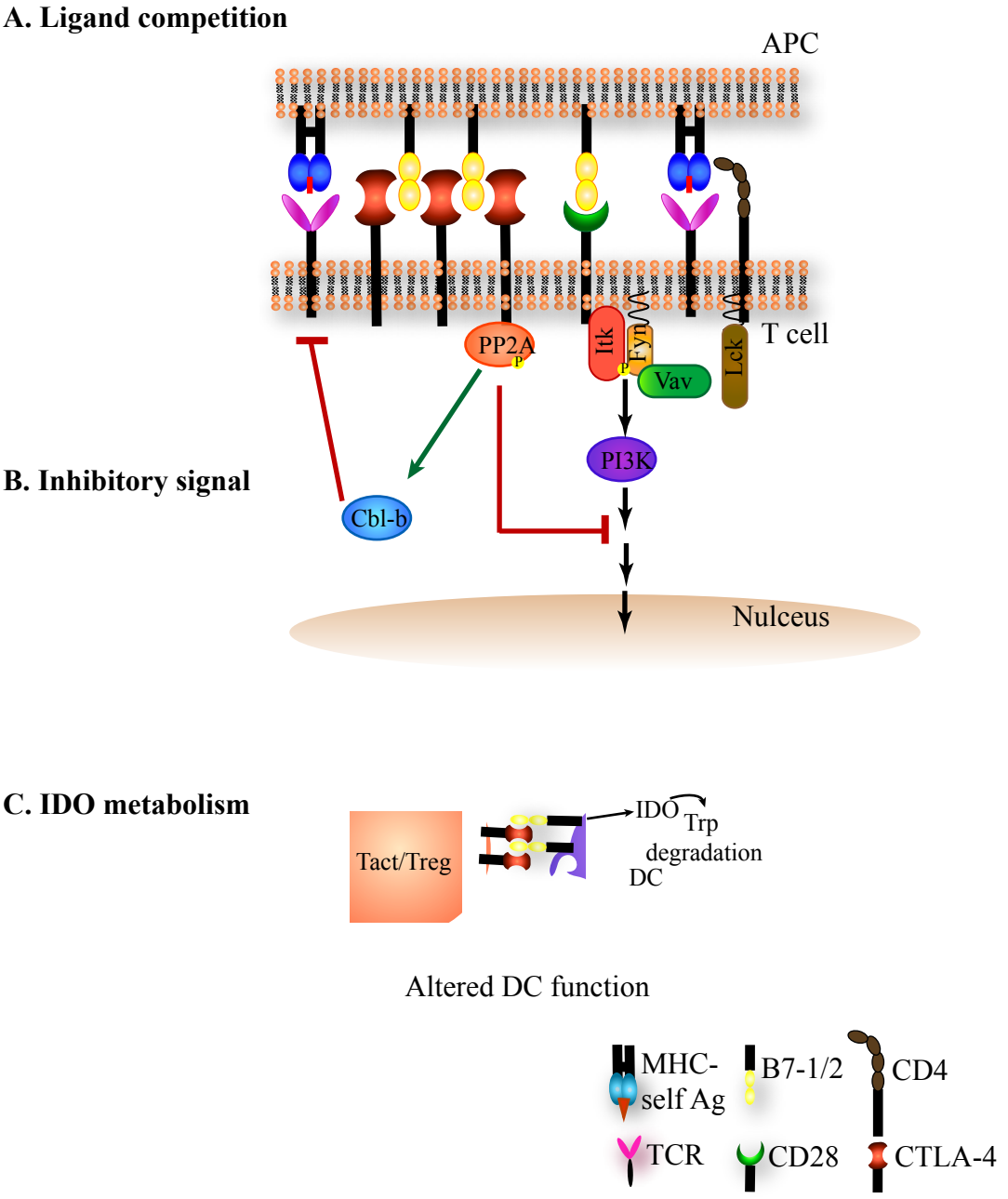

Figure I-4: Mechanisms of CTLA-4 regulated inhibition of T cell activation

Figure I-4: Mechanisms of CTLA-4 regulated inhibition of T cell activation

CTLA-4 is a negative costimulatory molecule and can inhibit T cell activation in several ways. Some of them are:

A. CTLA-4 can compete with CD28 for binding to the ligands B7-1 and B7-2 on antigen presenting cells. CTLA-4 binds to these ligands with higher affinity than CD28 and bivalent binding of CTLA-4 to B7 molecules can result in the formation of a lattice-like structure that can disrupt the immunological synapse.

B. CTLA-4 binding to B7 can initiate an inhibitory signal transduction pathway involving Cbl-b, which can inhibit TCR signals. Recruitment of phosphatases such as PP2A can also inhibit TCR and CD28 signals.

C. CTLA-4 expressed on Treg cells and/or activated T cells can generate a 'reverse' signal into APCs through B7 binding that leads to the production of indoleamine deoxygenase (IDO). IDO can catalyze the breakdown of tryptophan resulting in altered dendritic cell function and inhibition of T cell activation.

CD28 and CTLA-4 both bind the same receptors, B7-1 and B7-2 on antigen presenting cells. However, CTLA-4 binds to both these ligands with much higher affinity compared to CD28, with an even greater preference for B7-1. Therefore, in a ligand limiting condition, as is the case in homeostatic conditions or during the very early phases of inflammation, CTLA-4 will preferentially bind to B7 molecules depriving T cells of positive costimulation. Support for this ligand competition model comes from the fact that transgenic expression of a tailless form of CTLA4 (lacking the cytoplasmic domain) can completely ameliorate the fatal lymphoproliferative disease of *Ctla4*$^{-/-}$ mice (172, 211). While the lymphocytic infiltration into tissues that is characteristic of *Ctla4*-deficiency is absent, the mice do exhibit lymphadenopathy and increased T cell numbers. Some have taken this as evidence that CTLA-4 inhibits T cell activation to self-antigen by competing with CD28 for ligand binding, and thus inhibiting the delivery of a positive costimulatory signal.

While ligand sequestration is a simple model to explain CTLA-4 mediated inhibition of naïve T cell activation, its relevance *in vivo* is questionable and it is unlikely to be the primary cause of aberrant T cell activation seen in *Ctla4*$^{-/-}$ mice. Firstly, this model is dependent on surface CTLA-4 expression on naïve T cells, while naïve T cells *in vivo* barely express CTLA-4 mRNA, let alone surface CTLA-4 protein. Further, T cells in the tailless *Ctla4*Tg*Ctla4*$^{-/-}$ mice still display an activated phenotype and proliferate. So clearly, ligand competition with CD28 is not involved in the regulation of initiation of T cell activation. However, it may play a role in inhibiting CD28 signals that lead to tissue migration, and this is discussed in subsequent sections.

Inhibitory signaling

In addition to inhibiting the delivery of a positive costimulatory signal via CD28, CTLA-4 can also deliver an inhibitory signal though its cytoplasmic tail. Early studies showed that antibody cross-linking of CTLA-4 during T cell activation could inhibit IL-2 production and proliferation. However, these studies did not take into account the presence of Treg cells in the cultures that could inhibit T cell activation. Although one argument against the effect of Treg cells in these assays is that TCR transgenic T cells, that are not contaminated with Treg cells are also inhibited by CTLA-4 crosslinking, and more pronounced effects of antibody crosslinking are seen on previously activated T cells that express more CTLA-4 on their surface.

While the precise inhibitory signals delivered by CTLA-4 are not clear, it has been proposed to function at three levels: **a.** CTLA-4 inhibits early events in TCR signaling, **b.** CTLA-4 directly inhibits CD28 signaling and **c.** CTLA-4 inhibits downstream target of TCR and CD28 signals. In support of this, CTLA-4 has been shown to recruit phosphatases to the TCR complex and abort early TCR signaling events like ZAP-70 phosphorylation (212). The ligand-independent isoform of CTLA-4 can also bind to and dephosphorylate the TCRζ chain (180). Further, CTLA-4 signaling downregulates cytokine production by inhibiting the accumulation of AP-1, NF-κB and NFAT in the nucleus (213, 214).

Again, although a negative signal transduction pathway from CTLA-4 is an attractive model for inhibition of T cell activation by CTLA-4, it does not explain the

dysregulated T cell activation in *Ctla4*$^{-/-}$ mice. Naïve T cells lack surface CTLA-4 expression and since TCR signals are usually transmitted within the first few minutes to hours of initial T-APC interaction, it is difficult to envision how CTLA-4 could inhibit these early signaling events.

IDO/tryptophan metabolism

B7 molecules can control immune responses by reverse signaling into APCs. Ligation of B7-1 and B7-2 by CTLA-4, and possibly CD28, delivers a 'back' signal into APCs that activates an immunosuppressive pathway of tryptophan metabolism (215). Treatment of DCs with CTLA4Ig leads to the upregulation of IDO (indoleamine 2, 3-dioxygenase) via an IFNγ dependent pathway that utilizes the signaling adaptor DAP12 and the transcription factor IRF-8 (interferon regulatory factor-8) (216). IDO is responsible for the breakdown of tryptophan to byproducts that inhibit T cell proliferation. Therefore, one mechanism by which CTLA-4 could regulate immune responses is by modulation of APCs (217). While this model is attractive in that it can account for regulation of peripheral tolerance under homeostatic conditions via CTLA-4 expressing Treg cells, it is not clear how CTLA-4 ligation specifically, and not CD28, can lead to the induction of IDO, considering they both bind B7. Further, the pathway of suppression initiated by IDO is not well characterized, and whether this is a mechanism used by CTLA-4 on Treg cells or activated T cells is not understood.

Homing

CTLA-4 can also modulate T cell interaction with APCs, although the data in this regard is somewhat conflicting. It was shown that CTLA-4 could alter the expression of adhesion molecules such as LFA-1 (218). Consistent with this, CTLA-4 sufficient T cells adhere much more strongly to ICAM1 expressing substrates than CTLA-4 deficient T cells, suggesting that CTLA-4 promotes stable T-APC interaction. In contrast, CTLA-4 has also been shown to increase the motility of T cells when they are stimulated through their TCRs, such that these cells can only make short-lived contacts with APCs that results in abortive activation (219). One major caveat to the interpretation of these studies is that surface CTLA-4 is expressed very late in the T cell activation process, and it is unlikely that this altered interaction with APCs would influence early activation events. However, in a homeostatic environment, interactions between APCs and Treg cells, which constitutively express CTLA-4, might influence whether an immune response is induced.

CTLA-4 and Treg cells

Suggestions that CTLA-4 may function in a cell autonomous manner to regulate T cell activation came from early studies of mixed bone marrow (BM) chimeras of *wt* and *Ctla4*$^{-/-}$ BM. CTLA-4 sufficient *wt* BM could prevent the activation of *Ctla4*$^{-/-}$ T cells in these chimaeras and the mice could be maintained in a healthy state indefinitely (220). In un-manipulated mice, expression of CTLA-4 is mostly restricted to Treg cells. Therefore, one interpretation of the mixed BM chimera data is that CTLA-4 is primarily required for Treg cell function and/or maintenance and the lymphoproliferation observed in *Ctla4*$^{-/-}$ mice is primarily a consequence of defective Treg cells. The fact that the phenotype of *Ctla4*$^{-/-}$ mice closely resembles that of

Foxp3$^{-/-}$ mice supports this theory. However, unlike *Foxp3*$^{-/-}$ mice that completely lack Treg cells, *Ctla4*$^{-/-}$ mice do have FOXP3^{+} cells in peripheral lymphoid organs, suggesting that CTLA-4 is not required for the development of these cells in the thymus. Interestingly, transgenic expression of FOXP3 in *Ctla4*$^{-/-}$ mice can extend the lifespan of *Ctla4*$^{-/-}$ mice to up to 6 months (90) (Khattri R, NI 2003), suggesting that CTLA-4 may not be required for Treg cell function and that FOXP3 expression alone is sufficient. However, FOXP3 can regulate several genes involved in activation and cell cycle progression. Therefore, a rescue of phenotype of *Ctla4*$^{-/-}$ mice by over-expression of FOXP3 does not rule out a role for CTLA-4 in Treg function.

Until recently, the role of CTLA-4 in mediating Treg cell function was controversial. Adoptive transfer of naïve (CD25^{-}) CD4^{+} T cells into lymphopenic hosts rapidly leads to colitis unless FOXP3^{+} CD4^{+}CD25^{+} Treg cells are also transferred. Protection from colitis is abrogated by injection of blocking Ab against CTLA-4 suggesting that CTLA-4 is necessary for immune regulation in this model system (221). While the relevant target of Ab blockade (e.g. CTLA-4 on effector T cells or Tregs) in this model was unknown, it has been shown that CTLA-4 blockade variably disrupts the control of colitogenic B7-deficient (*Cd80*$^{-/-}$*Cd86*$^{-/-}$) *Clta4*$^{-/-}$ T cells by *wt* (CTLA4^{+}) Treg cells, suggesting that CTLA-4 on Treg cells is functionally relevant for initiating and/or maintaining regulation. However, the situation remained uncertain since experiments utilizing Ab-mediated blockade of CTLA-4 have given inconsistent results in the same model system and Treg cells from *Ctla4*$^{-/-}$ mice have also been reported to prevent the progression of colitis. The most conclusive demonstration so far about the role of CTLA-4 in Treg cell function

has come from the recent generation of conditional *Ctla4*$^{-/-}$ mice. Ablation of CTLA-4 in Treg cells by CRE expressed from a *Foxp3* promoter (Foxp3-Cre), results in a breakdown of tolerance similar to that in *Ctla4*$^{-/-}$ mice, leading to death of mice by 6-10 weeks of age (222). However, the phenotype of the mice does not faithfully phenocopy that of *Ctla4*$^{-/-}$ mice, and additional complexities and requirements of CTLA-4 in Treg cells and conventional T cells need to be investigated.

Transforming Growth Factor-beta (TGFβ) and peripheral tolerance

TGFβ is a plieotropic morphogen that has several functions in the immune system. One critical role for TGFβ is in the maintenance of peripheral T cell tolerance. The following sections outline this aspect of TGFβ function.

Morphogens and TGFβ

TGFβ belongs to a class of proteins called morphogens that primarily function in orchestrating developmental decisions in several species. Morphogens were first identified in the fruit fly, *Drosophila melanogaster*, in the early 20th century by Thomas Hunt Morgan. In 1969, Lewis Wolpert, in his famous French Flag Model, conceptualized how the concentration of morphogens in the environment could subdivide a tissue into domains of different gene expression (223). A working definition of a morphogen based on several years of study is that it is a substance that works in a concentration gradient dependent manner to specify cell fate and govern the pattern of tissue development by providing positional cues. Morphogens can be either transcription factors that directly affect gene expression, or secreted proteins that signal between cells. The first morphogen to be identified was Bicoid, a

transcription factor regulating the development of the *Drosophila* syncitial embryo. Another secreted morphogen described around the same time was Decapentaplegic, the product of a gene locus that governs the development of the 15 major imaginal discs in *D. melanogaster* larvae. The mammalian homologue of Decapentaplegic is the Transforming Growth Factor-beta (TGFβ), which plays pleiotropic roles in regulating cell proliferation, lineage determination, differentiation, motility, adhesion and death.

There are several TGFβ family members, and they can be divided into three major groups: TGF proteins themselves, bone morphogenetic proteins (BMPs) and Activin (224). In mammals three isoforms of TGFβ are expressed that can have positive and negative influences on a variety of cellular processes. These are TGFβ1, TGFβ2 and TGFβ3, with TGFβ1 being the most relevant for the immune system (225). However, TGFβ1 also plays a role in the regulation of differentiation and homeostasis of cardiomyocytes, pancreatic β-islet cells and blood vessel endothelium (226-228). TGFβ2 and 3 are predominantly expressed in mesenchymal tissues and bones where they have various functions in bone development and murine palate formation. The expression of TGFβ2 and TGFβ3 is mostly under the control of developmental or hormonal signals. TGFβ2 and TGFβ3 knockout mice are embryonic lethal and have major developmental defects resulting in malformation of bones and internal organs (229).

Cellular sources of TGFβ1

Tumor cells are major secretors of TGFβ1 (230), which regulates the tumor microenvironment partially by recruiting immune cells and coordinating their function (231, 232). Innate cells such as neutrophils, NK cells, monocytes and monocyte-derived macrophages also contribute to the accumulation of TGFβ1 in the tumor environment. Under homeostatic conditions, certain subsets of dendritic cells synthesize TGFβ. It has been reported that TGFβ1 produced by Peyer's Patch DCs and lung DCs can regulate the differentiation of effector T cells in these tissues and lead to either suppression or exacerbation of immune responses (233). Similarly, TGFβ1 secreted by tumor-infiltrating DCs also influences effector T cell lineage commitment. In addition, activated T cell subsets themselves produce TGFβ1 (234). As discussed previously, Th3 cells that are induced during the process of oral tolerance secrete TGFβ1, which then mediates immune suppression. $CD4^{+}Foxp3^{+}$ natural Treg cells also express surface TGFβ1 (235, 236); however whether this is relevant for their suppressive activity remains debatable.

TGFβ1 transcripts can be detected in a variety of lymphocytes including early thymic precursors such as DN2 and DN3, mature SP thymocytes and peripheral T cell subsets. However, in most cases, TGFβ1 message levels do not correlate with the amount of secreted protein, suggesting post-transcriptional and/or post-translational regulation of TGFβ1 expression (237). The transcriptional regulation of TGFβ1 expression is relatively unknown. The TGFβ1 promoter contains activator protein (AP-1) binding sites where c-jun and c-fos, induced by TGFβ itself can bind to and stimulate more TGFβ1 expression. Thus TGFβ1 can function in an auto-feedback loop and amplify its own production (229).

TGFβ signaling

TGFβ is synthesized as a pre-pro precursor protein. The pre region of the pre-pro-TGFβ precursor contains a signal peptide while pro-TGFβ is processed in the Golgi by a furin-like peptidase that removes the N-terminus of the immature protein (229). Homodimerization of this new protein, now called Latency associated protein (LAP), and subsequent noncovalent binding with a homodimer of mature TGFβ result in the formation of latent TGFβ or small latent complex (SLC). This complex can be secreted out by itself or in association with latent TGFβ-binding protein (LTBP) that plays a role in targeting TGFβ to the extracellular matrix. Proteolytic cleavage or a conformational change resulting in the release of TGFβ from LAP and LTBP allows mature TGFβ to bind to its receptors.

Biological effects of TGFβ1 are mediated by its binding to type I and type II trans-membrane serine/threonine kinase receptors (238). There are five type I receptors (also called activin receptor like kinase family ALK family), and seven type II receptors. TGFβ1 binds to ALK5 and TGFβ-receptor II (TGFβRII). TGFβRII exists in a basally phosphorylated state (239). Binding of mature TGFβ ligand to homodimeric TGFβRII results in the recruitment of TGFβRI and formation of a heterotetrameric complex. Ligand binding does not increase the phosphorylated state of TGFβRII, but allows it to catalyze the phosphorylation of TGFβRI at several serine and threonine residues. It is thought that TGFβRII acts as a constitutively active kinase, which upon ligand binding, can access its substrate, TGFβRI, and initiate signaling. SMAD (mothers against decapentaplegic homologue) proteins transduce

the signals from the TGFβRI to the nucleus. Although SMAD proteins bind weakly to DNA (240), their activity is mediated by interaction with a large number of transcriptional partners to regulate the TGFβ induced gene expression profile. There are also SMAD-independent signaling pathways that involve mitogen-activated protein kinase (MAPK), PI3K kinase, PP2A phosphatase and Rho proteins (241).

The activated TGFβR complex undergoes endocytosis via two pathways- one leading to accumulation of activated complexes in endosomes where SMAD activation takes place, and the other to caveolin-coated vesicles from where the complex is targeted for proteasome-dependent degradation regulated by SMURF (SMAD ubiquitylation regulatory factor) E3 ubiquitin ligases (239, 242). The balance between these two pathways affects the strength of the TGFβR signal and subsequent biological consequences.

Vertebrate TGFβ signal transduction involves eight SMAD proteins that can be divided into three functional classes: receptor associated SMADs or R-SMADs including SMAD1, SMAD2, SMAD3, SMAD5 and SMAD8; co-mediator SMADS or co-SMADs such as SMAD4; and inhibitory SMADs or I-SMADs such as SMAD6 and SMAD7. Only SMAD2 and SMAD3 can be activated by TGFβRI signaling (238). R-SMADs are associated with the membrane-anchored protein, SARA (SMAD anchor for receptor activation), which is abundant in endosomes where SMAD activation by TGFβRI takes place and also on the plasma membrane. R-SMAD phosphorylation/activation destabilizes its interaction with SARA, leading to the association of R-SMADs with SMAD4, followed by nuclear translocation. SMAD

proteins are rapidly dephosphorylated in the nucleus and shuttled back into the cytoplasm, thereby allowing for the dynamic regulation of TGFβ1 signaling to extracellular stimuli. Inhibitory SMADs suppress TGFβ signaling by two mechanisms- firstly by competing with R-SMADs for binding to TGFβRI and secondly by recruiting SMURF E3 ubiquitin ligases for the degradation of TGFβRI (243, 244). As mentioned earlier SMADs bind DNA very weakly. Coupled with the fact that the SMAD binding motif, CAGAC, is usually present only in a single copy in the promoter elements of SMAD-responsive genes, SMADs have to recruit additional co-factors and co-activators to regulate gene expression.

TGFβ and DAB2

DAB2 is an adaptor protein in the TGFβ signaling pathway and was initially identified in a screen for proteins involved in growth factor signal transduction in macrophages (245). There are three alternatively spliced products of *Dab2* mRNA: the full-length p96 isoform, the p93 isoform and the p67 isoforms. *Dab2* is expressed in several tissues such as heart, lung, liver, skeletal muscle and kidney (246). However, the precise expression pattern of *Dab2* and its isoforms in the immune system is not known.

DAB2, also known as DOC-2 (Differentially expressed in Ovarian Carcinomas-2), is the mammalian homologue of the *Drosophila* Disabled protein (247). It contains a phosphotyrosine interacting domain (PID or PTB) and a proline rich SH3-binding domain that mediate its role as an adaptor protein. DAB2 binds to both SMAD2 and SMAD3 via its N-terminal PTB domain. It can also bind to both

TGFβRI and TGFβRII and it has been suggested that DAB2 serves to bridge the TGFβR complex to the SMAD signaling pathway (248). In addition, DAB2 can also associate with the mitogen activated kinase kinase kinase (MAPKK), TAK1, which can trigger the SMAD-independent TGFβR signaling pathway through JNK activation (249).

TGFβ and T cell development

TGFβ regulates the development and function of immune cells at multiple stages (**Figure I-5**). Early studies investigating the role of TGFβ in T cell development primarily relied on the use of *in vitro* cultures and fetal thymic organ cultures (FTOCs). First, TGFβ was shown to inhibit the IL-7 induced proliferation of total thymocytes *in vitro* (250, 251). TGFβ could also induce CD8 expression on $CD25^{+}$TN ($CD3^{-}CD4^{-}CD8^{-}$) precursor cells, suggesting that it may be involved in the regulation of thymic $CD8^{+}$ T cells (252). In addition, TGFβ1 produced by thymic epithelial cells could regulate the differentiation of $CD4^{-}CD8^{lo}$ αβ precursor cells into $CD4^{+}CD8^{+}$ DP thymocytes (253). TGFβ also inhibits the differentiation of precursor cells into $CD4^{+}CD8^{+}$ DP cells with a more pronounced inhibition of CD4SP cell generation. CD8SP cells can develop normally in the presence of TGFβ, however these cells have higher expression of CD8β, TCRβ and CD3 (254). While these were indications that TGFβ does influence T cell development, additional insights were only gained once the TGFβ1 knockout mice were made.

The first study of a *Tgfβ1*$^{-/-}$ mouse reported intrauterine lethality of ~50% of *Tgfβ1*$^{-/-}$ mice (reaching 100% on the C57/BL6 background) (255, 256). This is not

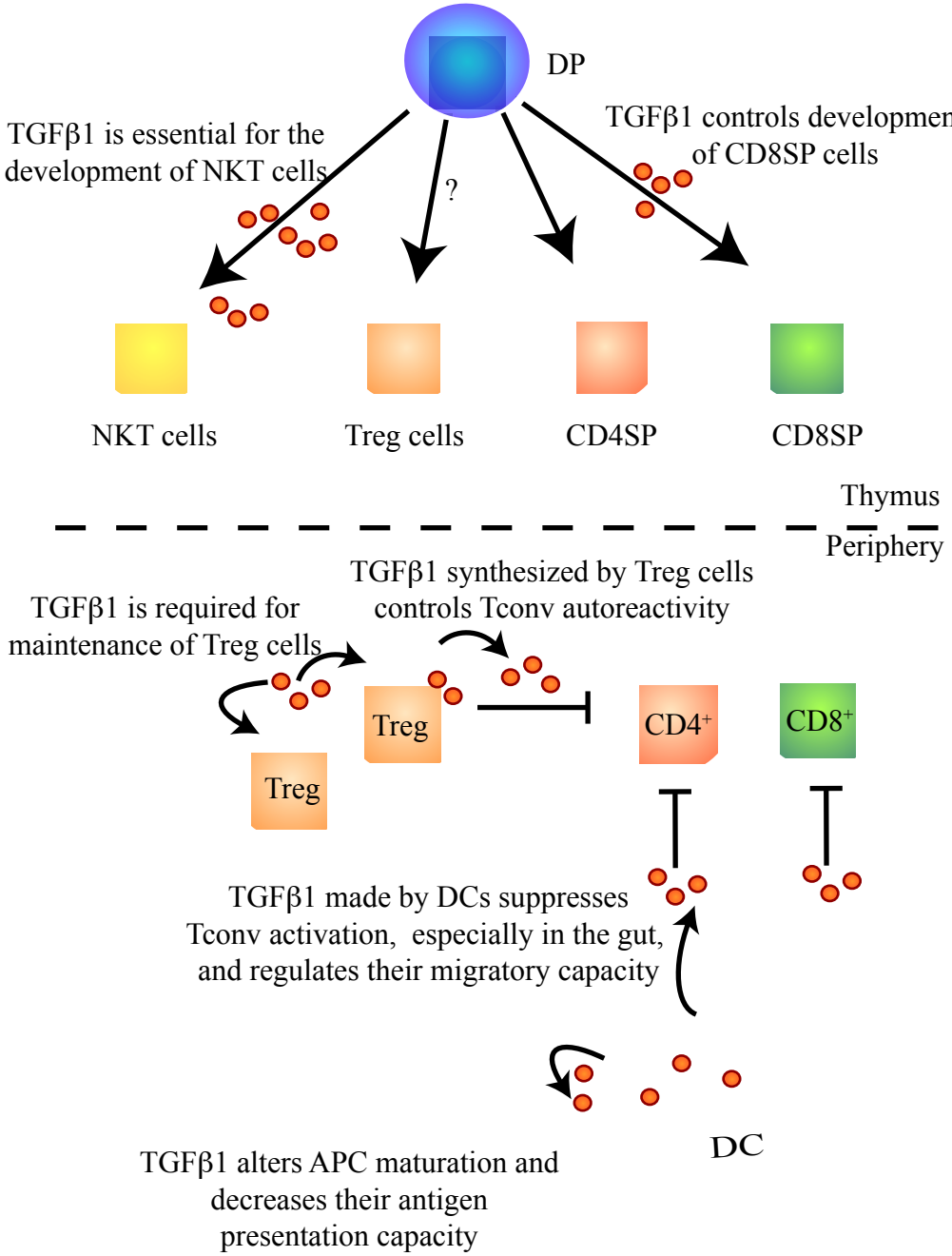

Figure I-5: TGFβ1 regulates the development and function of immune cells

Figure I-5: TGFβ regulates the development and function of immune cells.

TGFβ is a plieotrpic morphogen than regulates several aspects of immune cells development and function. In the thymus, TGFβ is required for the development of NKT cells and CD8SP cells. The role of TGFβ in thymic Treg cell development remains questionable.

In the periphery, TGFβ is required for the homeostatic maintenance of Treg cells. TGFβ produced by Treg cells, dendritic cells and non-immune cells is required for the control of conventional $CD4^+$ and $CD8^+$ T cell autoreactivity. TGFβ made by dendritic cells is also crucial for regulating homeostasis in the gut as well as for the control of APC function and antigen presentation capacity.

surprising because TGFβ1 is required for a number of developmental processes, including haematopoiesis (257). However, the few mice that are born have a severely attenuated life span and survive only until 3-5 weeks of age (255, 258). A rapidly progressing, spontaneous autoimmunity beginning at around 17 days of age results in massive $CD4^+$ T cell activation (225, 259, 260), lymphocyte infiltration and death of these mice. This disease can be prevented by crossing the *Tgfβ1*$^{-/-}$ mice with SCID mice (260) or MHC-class II deficient mice (261); suggesting that T cells are the primary mediators of autoimmunity in *Tgfβ1*$^{-/-}$ mice. Because of the defects in multiple cell subsets in *Tgfβ1*$^{-/-}$ mice, the precise role of TGFβ1 in the immune system still remained unclear.

To investigate this, transgenic mice that express a dominant-negative TGFβRII from a T cell specific promoter that abrogates all TGFβ (1,2 and 3) signaling in T cells were generated. Two promoters were used, *Cd4* (CD4-DNRII) (262) and *Cd2* (CD2-DNRII) (263), that surprisingly, gave distinct results. The CD4-DNRII mice survive to adulthood, but they develop an inflammatory disease characterized by organ infiltration and presence of circulating autoantibodies. The presence of activated/memory $CD4^+$ T cells, in conjunction with previous observations with MHC-Class II deficient-*Tgfβ1*$^{-/-}$ mice, led to the conclusion that $CD4^+$ T cells and not $CD8^+$ T cells are the likely mediators and initiators of inflammation in *Tgfβ1*$^{-/-}$ mice. Contrary to this, the disease in CD2-DNRII mice is mediated by $CD8^+$ T cells that proliferate indiscriminately, probably at the expense of $CD4^+$ T cells, leading to enlarged secondary lymphoid organs. Interestingly, this $CD8^+$ lymphoproliferation is not associated with typical inflammation. In both models

however, thymocyte development is not affected. Several differences in the two models could account for this distinct phenotype. Firstly, the two promoters are expressed at different stages during T cell development. CD2 is expressed in early DN2 precursor cells resulting in abrogation of TGFβ signaling much earlier in ontogeny than CD4, which is expressed only at the DP stage of αβ T cell development. Thus, expression of the transgene in early precursors could potentially affect TGFβ responsiveness of multiple T cell lineages including NKT cells and γδ T cells. Further, it is possible that TGFβ signaling in precursor αβ T cells results in distinct developmental outcomes compared to TGFβ signaling in more mature thymocytes. In addition, the strength of the promoters may also vary, resulting in residual TGFβRII signaling in the weaker promoter model. Indeed, addition of exogenous TGFβ to $CD4^{+}$T cells from CD4-DNRII mice *in vitro*, led to low but detectable nuclear translocation of SMAD2 and SMAD3 (264), suggesting some TGFβRI signaling in these T cells.

The first clear-cut evidence that TGFβ1 is essential for T cell development and homeostasis came from the generation of conditional TGFβRII knockout mice by two independent laboratories (264, 265). In both reports, deletion of TGFβRII using CD4Cre results in the lack of expression of TGFβRII in DP thymocytes and CD4 and CD8SP cells in the thymus and periphery. The phenotype of the CD4Cre:$TGF\beta RII^{-/-}$ mice recapitulates that of $Tgf\beta1^{-/-}$ mice, and the mice die by 3-5weeks of age from severe inflammation. Both CD4 and CD8 T cells display an activated phenotype that precipitates into autoimmunity affecting multiple organs. However, while one study found a two-fold reduction in the frequency and numbers of conventional mature

$CD8^+$T cells (264), the other study reported normal $CD8^+$ T cell development (265). The analysis of thymocyte development in mice lacking any aspect of TGFβ signaling is complicated by the severe immunopathology that can potentially affect T cell development. Thus, although both reports utilized young mice that do not display overt inflammation, differences in the two studies may result from subtle alterations in chemokine/cytokine patterns. To circumvent this problem, Ming Li has generated TGFβRII knockout mice on the H-Y TCR transgenic mouse background. Unpublished observations from this mouse model cited in (266) suggest that, in the absence of inflammation, $CD8^+$H-Y Tg T cells are attenuated in the thymus and TGFβ signaling may play a role in promoting $CD8^+$T cell development. Further, the use of TCR transgenic mice carrying the TGFβRII mutation has revealed a role for TGFβ in maintaining naïve T cell homeostasis. This observation comes from the fact that on a TCR transgenic background, TGFβ signaling deficient T cells can be indefinitely maintained in a naïve state (265, 267, 268). Further, a study of the TCR diversity in TGFβ1 deficient mice revealed that peripheral but not thymic $CD4^+$T cells exhibited a nonpolyclonal distribution of the third complementarity determining region (CDR3) of TCR Vβ chains, suggesting death/loss of specific $CD4^+$T cells in peripheral lymphoid organs (269). Thus, TGFβ1 maintains T cell homeostasis by regulating naïve T cell activation, proliferation and death.

TGFβ1 also affects the development of CD1d restricted NKT cells in the thymus and CD4Cre:*TGFβRII*$^{-/-}$ mice have sharply reduced thymic and peripheral NKT cell numbers. In addition, blockade of TGFβ signaling results in the

development of a highly pathogenic T cell subset that exhibits characteristic features of NK cells that have elevated FasL, perforin, granzyme and IFNγ expression (265).

TGFβ and natural Treg cells

Since the *Tgfβ1*$^{-/-}$ mice closely resemble the *Foxp3*$^{-/-}$ mice, it has been suggested that the loss of peripheral tolerance in *Tgfβ1*$^{-/-}$ is due to the lack of functional Treg cells. However, TGFβ signaling appears to be dispensable for Treg cell generation because two week old CD4Cre:*TGFβRII*$^{-/-}$ mice have normal perhaps even moderately higher frequencies of CD4^{+}FOXP3^{+} cells in the thymus. However, the frequency of Treg cells in the spleen is significantly reduced, which could be due to improper maintenance of *TGFβRII*$^{-/-}$ Treg cells in the periphery, or because of massive expansion of conventional *TGFβRII*$^{-/-}$ CD4^{+}T cells. Surprisingly, it was found that FOXP3^{+} cells in the spleen of CD4Cre: *TGFβRII*$^{-/-}$ mice incorporated more BrdU than littermate controls, suggesting an increased turnover rate of TGFβ signaling deficient Treg cells (264). This suggests that TGFβ may be essential for the survival and maintenance of Treg cells in the periphery, but may not be required for their development in the thymus. Contrary to this, another study that analyzed the phenotype of 3-5 days old neonatal LckCre:*TGFβRII*$^{-/-}$ mice (LckCre mediates deletion of TGFβRII in early DN2 cells in the thymus) showed a two-fold reduction in the frequency of FOXP3^{+} Treg cells in the thymus (270). However, as the mice age, there is a rapid increase in the frequency and numbers of Treg cells in the thymus, which is dependent on the increased IL-2 concentration in the thymus of LckCre:*TGFβRII*$^{-/-}$ mice. In addition, the Treg cells from LckCre:*TGFβRII*$^{-/-}$ mice are not functional and cannot suppress the proliferation of responder T cells *in vitro*.

While the role of TGFβ in regulating Treg cell development and functionality remains uncertain, the development of autoimmunity in TGFβRII conditional knockout mice is probably not due to defective Treg cells. Transfer of *wt* Treg cells into neonatal CD4Cre: *TGFβRII*$^{-/-}$ mice cannot prevent the activation of TGFβRII deficient CD4^{+}T cells or the autoimmune pathology, despite the robust maintenance of the *wt* Treg cells in these mice (264). These findings are consistent with an earlier study that showed that *wt* Treg cells could not control colitis induced by naïve CD4-DNRII CD4^{+}T cells in a mouse model of inflammatory bowel disease (IBD) (271). Mixed bone marrow chimera experiments also revealed that TGFβRII deficient T cells always exhibit a more activated phenotype than *wt* T cells (264, 265), suggesting that TGFβ may promote T cell tolerance by directly regulating both conventional and regulatory T cell populations.

TGFβ and induced Treg (iTreg) cells

Another important mechanism of TGFβ mediated immune suppression is by promoting the generation of Treg cells in the periphery. Following TGFβ stimulation, regulatory activity can be induced in human naïve CD4^{+}CD45RA^{+} T cells (272). Importantly, TGFβ can induce *de novo* expression of FOXP3 in naive conventional CD4^{+} T cells and confers suppressive function to these induced Treg cells (95, 97, 98). Induction and maintenance of FOXP3 in iTreg cells requires high concentrations of TGFβ. This dose dependent regulation of immune effector responses is an important aspect of TGFβ biology. An elegant study showed that high concentrations of TGFβ is required to commit to the FOXP3^{+} Treg lineage and low concentrations of

TGFβ lead to the generation of an alternate T cell subset producing pro-inflammatory cytokines such as IL-17 (273). In support of this, over-expression of TGFβ1 in islet cells of the pancreas causes the expansion of $FOXP3^+$ Treg cells that protect NOD mice from diabetes (274). One *in vivo* environment that is characterized by high TGFβ1 concentration is the gut-associated lymphoid tissue (GALT). This mucosal niche is an important site for the generation if iTreg cells. Transfer of naïve polyclonal $CD4^+FOXP3^-$ T cells into congenic *wt* recipients results in the conversion of a subset of these cells to iTreg cells that accumulate in the GALT (275). Further, $CD103^+$ DCs have been implicated in assisting in this conversion to iTreg cells. Retinoic acid, a Vitamin A metabolite that is produced in high amounts by these DCs is responsible for this conversion, and also for the induction of gut-homing receptors such as α4β7 integrin and CCR9 on T cells (276). The role of retinoic acid in regulating iTreg cell conversion is discussed in greater detail below. Endogenous TGFβ at sites other than the mucosa can also induce expression of FOXP3 in conventional T cells. Steady-state dendritic cells pulsed with low doses of agonist peptide ligands can induce *de novo* generation of Treg cells from naïve T cells. This conversion is inhibited in CD4-DNRII mice in which T cells lack TGFβR signaling and display hyperproliferation (277). Since proliferation seems to be inversely correlated with the induction of FOXP3, one mechanism by which TGFβ could assist in the induction of FOXP3 is by limiting T cell division.

The molecular mechanism by which TGFβ1 regulates *Foxp3* expression is still not fully understood. TGFβ1 signaling in T cells activates the R-SMADs, SMAD2 and SMAD3, which together with co-SMAD, SMAD4, translocate to the nucleus to

regulate gene expression. Development of FOXP3^{+} Treg in *Smad3*$^{-/-}$ mice has been difficult to study because of the lethal colorectal adnenocarcinomas that develop in these mice at 4-6 months of age (278). However, another *Smad3* knockout mouse generated by the disruption of *exon8*, revealed a distinct phenotype. The disruption of *Smad3* was primarily associated with impaired mucosal immunity and massive T cell activation and proliferation. Further, these T cells had diminished responsiveness to TGFβ mediated proliferative arrest (279). Although the role of *Smad3*$^{-/-}$ Treg cells in regulating T cell homeostasis was not addressed then, more recent evidence points to an important function of SMAD3 in inducing FOXP3 expression in Treg cells (115). TGFβ1 treatment of TCR and CD28 stimulated naïve T cells delivers a signal that results in recruitment of SMAD3 and NFAT to a conserved binding site on the *Foxp3* enhancer region. Synergistic action of SMAD3 and NFAT is required for histone H4 acetylation of the enhancer region and initiation of *Foxp3* transcription. SMAD2 and SMAD4 do not associate with the enhancer region and are not required for *Foxp3* expression in this particular system. Interestingly, however, defects in TGFβ signaling have no apparent effect on intrathymic development of FOXP3^{+} Treg cells. This is at odds with the requirement of TGFβ signaling intermediate, SMAD3, for *Foxp3* expression. One explanation is that additional TGFβ family members such as Activins and BMPs could initiate signaling through the SMAD pathway and activate SMAD3 leading to *Foxp3* expression in TGFβ1 knockout or TGFBRII conditional knockout mice. There may also be SMAD3 independent pathways that could result in *Foxp3* expression in TGFβ1 deficient mice.

SMAD4 has also been implicated in regulating *Foxp3* expression upon TGFβ stimulation (280). Unlike *Smad3*$^{-/-}$ mice, *Smad4*$^{-/-}$ mice are embryonic lethal and die around embryonic day 6.5 (E6.5) due to lack of gastrulation (281, 282). Surprisingly, mice with a conditional deletion of SMAD4 in T cells by Cre-recombinase expression from the *Cd4* promoter are healthy and have normal thymic αβ T cell development (280). This is incongruent with the fact that TGFβ signaling is critical for normal thymic development and that SMAD4 is an important transducer of TGFβ signals into the nucleus. In addition, the development of thymic derived FOXP3^{+} Treg cells in CD4Cre:*Smad4*$^{fl/fl}$ mice is also normal and these Treg cells appear to be functional in an *in vitro* suppression assay. Of interest is the fact that upon TGFβ1 treatment *in vitro*, *Smad4*$^{-/-}$ T cells do not convert as efficiently to the FOXP3^{+} Treg lineage as *wt* T cells. Their ability to convert to IL-17 producing Th17 cells, however, is normal. This suggests that SMAD4 may play a role in regulating *Foxp3* expression upon TGFβ stimulation, but may be dispensable for Th17 lineage differentiation.

TGFβ and helper T cell differentiation

Upon activation, CD4^{+} T cells differentiate into helper or effector T cells that can secrete different cytokines and chemotactic factors that mobilize other host cells to mount an immune response to specific invading pathogens (**Figure I-6**). There are several effector T cell lineages, which are characterized by their ability to produce signature cytokines. The best described helper T cells are the Th1 cells that secrete IFNγ and lymphotoxin to activate the cellular arm of adaptive immunity in response to intracellular pathogens, and the Th2 cells that produce IL-4, IL-5 and IL-13 and direct antibody production by B cells to extracellular pathogens. The differentiation of

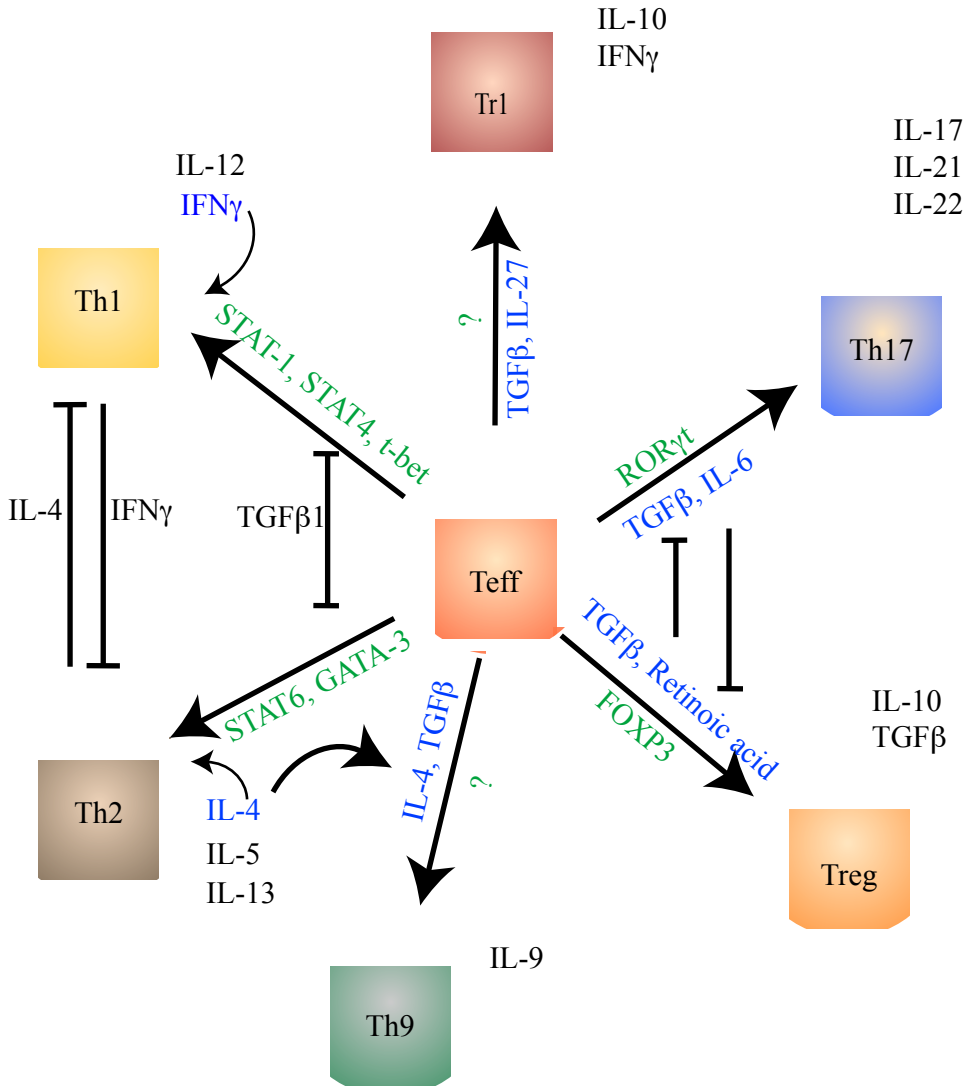

Figure I-6: TGFβ controls effector T cell differentiation

Figure I-6: TGFβ controls effector T cell differentiation

The differentiation of naïve $CD4^+$T cells into effector lineages requires the concerted effort of skewing cytokines and growth factors (blue text) as well as the expression of specific transcription factors (green text). TGFβ can cooperate with other cytokines and orchestrate this differentiation, resulting in either the suppression of immune responses or the generation of an inflammatory response.

TGFβ inhibits the differentiation of both Th1 and Th2 cells. However, in the presence of IL-4, TGFβ can direct the conversion of T cells into IL-9 producing Th9 cells. TGFβ alone or in concert with retinoic acid can differentiate naïve T cells into FOXP3 expressing Treg cells, but TGFβ in the presence of IL-6 can convert naïve T cells into IL-17 producing Th17 cells. TGFβ and IL-27 facilitates the development of Tr1 cells that produce IL-10 and IFNγ. Thus, TGFβ is central to the differentiation of several effector T cel lineages and can dictate the quality of the immune response that is generated in response to pathogenic stimuli.

naïve T cells into these effector cells requires the expression of specific transcription factors that commit activated T cells to one lineage or the other. For Th1 cells, this is T-bet and STAT1/4, and for Th2 cells, it is GATA-3 and STAT6 (283).

A new lineage of helper T cells called Th17 cells that produced IL-17A and Il-17F was described to develop from a pathway distinct from Th1 and Th2 cells (284). More interestingly, while TGFβ is vital for the induction of FOXP3 in naïve T cells, differentiation of Th17 cells from naïve $CD4^+$ T cells also requires TGFβ (285-287). This reciprocal regulation of the generation of anti-inflammatory Treg cells and pro-inflammatory Th17 cells by TGFβ is central to the outcome of several autoimmune diseases and infections. In addition to TGFβ signals, the differentiation of naïve $CD4^+$ T cells to the Th17 lineage requires IL-6, a pro-inflammatory cytokine produced by innate cells upon stimulation via Toll-like receptors and C-type lectin receptors. Thus in the absence of inflammation, TGFβ induces the generation of $FOXP3^+$ Treg cells, while IL-6 produced during inflammation tips the balance towards IL-17 producing Th17 cells and also actively inhibits the generation of Treg cells (285). IL-21 is another cytokine that can induce Th17 cells- however, TGFβ plus IL-6 treatment of IL21R-deficient T cells does induce the conversion of a small frequency of cells to Th17 lineage. Additionally, Th17 cells themselves produce copious amounts of IL-21, and it has therefore been suggested that IL-21 serves to reinforce the Th17 phenotype by amplifying IL-17 production (288-290). The role of IL-23 in Th17 cell differentiation has very recently been clarified. Contrary to initial observations that IL-23 could differentiate Th17 cells (291), it is now thought that IL-23 binding to its

receptor IL-23R, which is induced by IL-6 and IL-21 on activated T cells (290), is required for the maintenance of Th17 cells (292).

The transcription factor RORγt is necessary and sufficient for differentiation of Th17 cells (293). In addition STAT3 activation by IL-6 signaling is also necessary for IL-17 production, and conditional deletion of *Stat3* in T cells prevents the generation of Th17 cells (294). The regulation of iTreg cell versus Th17 cell generation at the transcriptional level is only now being elucidated. Interestingly, TGFβ stimulation can induce expression of both FOXP3 and RORγt simultaneously in TCR stimulated T cells *in vitro* (273). In fact, these double positive cells can also be found in the lamina propria tissue of the small intestines, and further investigations have suggested that $FOXP3^{+}$ T cells can be differentiated into Th17 cells by pro-inflammatory cytokines. On the contrary, if only TGFβ is present, FOXP3 can directly bind to RORγt and repress its transcriptional activity. In addition, DNA binding activity of FOXP3 is also essential for the inhibition of *Il17* transcription suggesting a RORγt independent pathway of regulation by FOXP3. In the presence of IL-6, the inhibitory effects of FOXP3 on RORγt expression are relieved, perhaps by a STAT3 dependent mechanism. More recently, RUNX1 was shown to regulate *Il17* transcription by inducing RORγt expression. RUNX1 further physically associated with RORγt on a CNS region of the *Il17* promoter and initiated *Il17* transcription (295). On the other hand, RUNX1 also bound FOXP3 and this interaction was important for the negative effect of FOXP3 on Th17 differentiation. Thus, RUNX1-FOXP3 complex acts as a transcriptional repressor of *Il17* expression and RUNX1-

RORγt acts as a transcriptional activator, indicating that RUNX1 is an important transcription factor that positively and negatively regulates IL-17 expression.

TGFβ and Retinoic acid

The intestinal microenvironment is unique in that it is constantly exposed to foreign food-borne antigen and the requirement of regulatory mechanisms controlling tolerance is probably greatest in this tissue. Intestinal epithelial cells are a rich source of TGFβ, which can, as discussed above, influence the generation of suppressive iTreg cells versus pro-inflammatory Th17 cells. It has been shown that the intestine, and lymphoid tissue associated with the intestines such as the mesenteric lymph nodes (MLN) and the lamina propria (LP) are preferred sites for the generation of iTreg cells (275, 296, 297). However, the constant exposure of immune cells to luminal antigens creates a chronic inflammatory environment, which should favor the generation of Th17 cells. Indeed, the lamina propria is one environmental niche that contains IL-17 producing cells even under homeostatic conditions (273). Nevertheless, under normal steady-state conditions, the intestines are not inflamed and immune responses are under control, primarily because iTreg cells tend to accumulate at this site. The Vitamin A metabolite, Retinoic Acid (RA), was found to be crucial for this balance, and it was shown that RA could inhibit TGFβ plus IL-6 driven conversion of cells to the Th17 lineage, and promote the generation of iTreg cells (275, 296, 297).

The human body does not synthesize Vitamin A, and it is absorbed from the diet and metabolized to RA by various enzymes including alcohol dehydrogenases

(ADH) and retinal dehydrogenases (RALDH) (298). Of the isoforms of RA, only the *all-trans* retinoic acid (ATRA) is found *in vivo*, which can bind to nuclear receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs). These receptors can function as ligand-induced transcription factors that bind DNA at RA response elements (RARE). In addition to regulating iTreg and Th17 cell differentiation, RA can also influence IgA production at mucosal surfaces by regulating class switching. RA also induces migration and homing of B cells to the LP of small intestine. RA produced by MLN and Peyer's Patch (PP) DCs can induce expression of gut homing receptors, $\alpha 4\beta 7$ and CCR9 on T cells. In conjunction with TGFβ, RA can also regulate expression of the $a_E$integrin CD103 on T cells.

Thesis Objectives and Summary

One of the main challenges for the immune system is to ensure that auto-reactive T cells are kept in check in the periphery, and concomitantly, allow these cells to react to foreign antigen in an appropriate manner. T regulatory cells are critical regulators of this balance that prevents autoimmunity and allows the generation of a productive immune response. An understanding of the mechanisms utilized by Treg cells in maintaining this balance is useful from a clinical perspective in developing therapeutic and vaccination strategies for cancers and autoimmune diseases.

The major goal of my thesis research has been to understand the role of CTLA-4 and TGFβ in orchestrating Treg cell mediated regulation of peripheral tolerance. The data from these two studies are described in the following chapters.

Chapter II: CTLA-4 functions in Treg cells and conventional cells to prevent fatal autoimmunity.

Based on the expression pattern of CTLA-4 in T cells, we can predict that CTLA -4 could exert immune regulation at three distinct phases of the immune response. **1.** As naïve T cells enter cell cycle after TCR/CD28 signals, CTLA-4 induction could moderate the threshold and magnitude of the T cell response. **2.** CTLA-4 could act after T cells are fully activated to moderate clonal expansion and effector functions. **3.** CTLA-4 on Treg cells could inhibit naïve T cell activation in a non-inflamed environment. To determine when and where CTLA-4 is required to maintain peripheral tolerance, we have generated mouse models of restricted CTLA-4 expression. Analysis of these mice reveal a critical function for CTLA-4 in Treg cells

to maintain peripheral homeostasis, as well as a function of CTLA-4 on conventional T cells to prevent autoimmune migration and tissue destruction.

Chapter III: DAB2, a TGFβ signaling intermediate, is important for Treg cell function and for TGFβ responsiveness of conventional T cells.

In the search for FOXP3 target genes that may be important for Treg cell function, we have identified *Dab2*, a TGFβ signaling adaptor protein, as a molecule that is expressed exclusively in Treg cells in peripheral lymphocytes. We have investigated DAB2 function in Treg cells by utilizing conditional Dab2 deficient mice. Analysis of different conditional deletions of *Dab2* suggests that DAB2 functions at two stages to regulate T cell responsiveness and tolerance. First, DAB2 expression in Treg cells is important to maintain normal Treg cell function in vivo and in vitro. Second, DAB2 functions in thymic precursor cells to program the TGFβ responsiveness of mature conventional T cells.

CHAPTER II

CTLA-4 functions in Treg cells and activated T cells to prevent multi-organ autoimmunity

Attributions and Copyright Information

Dr. Hai Nguyen generated the *Il2pCtla4*Tg and the *flCtla4*Tg mice.

Much of the data described here is part of a manuscript that has been submitted for publication.

Introduction

Adaptive immunity requires T cell responses to foreign pathogens to be counterbalanced with the need to limit collateral destruction of the host's own tissues. A pivotal balancing act involves negative co-stimulation, or co-inhibition, impacting peripheral T cells. Normally, naïve T cell activation requires signals transmitted by the TCR and the positive costimulatory molecule CD28 (153). However, costimulation is much more intricate than initially envisioned and an integration of stimulatory and inhibitory signals over the course of T cell activation determines the ultimate outcome of initial T cell encounter with antigens. A pair of homologous immunoglobulin-like receptors, CD28 and CTLA-4, best illustrates the dynamic interplay of costimulatory molecules. Although both recognize B7 ligands on antigen presenting cells (APCs) (161, 299), CD28 enhances T cell responses while CTLA-4 limits them (187). B7 expression on APCs increases with inflammation (154) and it has been determined that CD28 is necessary for optimal naïve T cell activation and trafficking (300) coincident with the functional maturation of APCs while CTLA-4 has been proposed to moderate T cell activation to prevent unchecked continuation of T cell immune responses (187).

There exists a firm consensus on CD28 function, but when and where CTLA-4 acts to rein in T cell activation *in vivo* has been more elusive. There are two main reasons for this uncertainty. First, animal models to study CTLA-4 function during different phases of T cell activation and in distinct T cell subsets are lacking. The severe autoimmune lymphoproliferative disease of *Ctla4*$^{-/-}$ mice clearly demonstrated the importance of CTLA-4 in regulating T cell responses and pointed to a dominant

role in naïve T cell homeostasis and tolerance to self (169, 301). But the dysregulation of T cells soon after birth and death of *Ctla4*$^{-/-}$ mice at 3-4 weeks of age did not permit a detailed dissection of CTLA-4 function. Second, unlike in conventional CD4^{+} T cells, CTLA-4 is constitutively expressed on FOXP3^{+}CD4^{+} regulatory T (Treg) cells (191), in part because FOXP3 directs CTLA-4 transcription (121). Since FOXP3^{+} Treg cells dominantly control T cell self-tolerance (89) and Treg cell-deficient mice resemble *Ctla4*$^{-/-}$ mice in autoimmune disease progression (83), it has been speculated that the primary function of CTLA-4 is to affect Treg cell-mediated tolerance induction (191, 302). Consistent with this model we have shown that CTLA-4-expressing Treg cells are necessary and sufficient to control *Ctla4*$^{-/-}$ T cell activation *in vivo* (Friedline R in press). However, this result did not rule out additional functions of CTLA-4 in other T cell subsets during T cell activation, and the relative importance of CTLA-4 signaling *in cis* (conventional T cell intrinsic) versus *in trans* (via Treg cells) in maintaining T cell homeostasis remains undefined.

Theoretically, there are three distinct phases of T cell activation when CTLA-4 signaling can impose regulation: **1.** As naïve T cells enter cell cycle after TCR/CD28 signals, CTLA-4 induction can moderate the threshold and magnitude of the T cell response. *In vitro,* we estimate this window of activity to be within 8-12 hours after TCR engagement, after which point, T cells are committed to full activation (Jain N. and Chambers, C., unpublished observations). Some *in vitro* studies support the early function of CTLA-4 in T cell activation in the context of maximal TCR/CD28 signal induction (303). However, kinetics of induction of CTLA-4 *in vivo* (185, 304, 305) is not compatible with CTLA-4 action within a few

hours after TCR engagement. **2.** CTLA-4 acts after T cells are fully activated to moderate clonal expansion and effector functions (187). The majority of experimental data involving CTLA-4 manipulations in conventional T cells support this dampening effect of CTLA-4 subsequent to T cell activation. **3.** CTLA-4 on Treg cells inhibits naïve T cell activation in non-inflamed environment. In T cell transfer models of colitis, blocking CTLA-4 on Treg cells using mAb results in the impairment of Treg cell-mediated prevention of colitis (191, 302, 306), and CTLA-4^{+} Treg cells can inhibit *Ctla4*$^{-/-}$ T cell activation *in vivo* (Friedline, R. et al. manuscript in press). These results indicate that CTLA-4 is essential for some aspects of Treg cell function.

To determine when and where CTLA-4 functions, viable mouse models where CTLA-4 expression can be temporally and spatially controlled are required. Here, we tested the activity of CTLA-4 in conventional T cells by generating mice expressing functional CTLA-4 in activated T cells, but not in Treg cells. This model was created using a CTLA-4 transgene (Tg) whose expression is driven by the *Il2* promoter in *Ctla4*$^{-/-}$ mice. CTLA-4-less Treg cells were unable to inhibit aberrant naive T cell activation. Critically, while activated T cell-restricted CTLA-4 expression cannot prevent lymphoproliferation, it is sufficient to prevent activated T cell accumulation in non-lymphoid organs, thereby extending the life span of *Ctla4*$^{-/-}$ mice to over a year. There are two tissues not protected by activated T cell restricted CTLA-4 expression: the pancreas and gut. A late-onset enteritis eventually contributes to the death of the animals. Collectively, these results verify the functional link between CTLA-4 and Treg cells in the maintenance of T cell tolerance to self or environmental

antigens, and reveal a function for CTLA-4 in conventional T cells to regulate the accumulation of aberrantly activated T cells in non-lymphoid tissues.

RESULTS

Il2 promoter driven CTLA-4 expression is restricted to activated T cells.

To determine spatial and temporal requirements of CTLA-4 in T cell subsets, we generated a transgenic mouse model of restricted CTLA-4 expression. Since the induction of *Il2* gene transcription is tightly regulated and is one of the earliest events after T cell activation (104), we chose to use the *Il2* promoter to drive a burst of CTLA-4 expression only at the initial phase of T cell activation, but not in naïve T cells. We generated CTLA-4 Tg mice, designated *Il2pCtla4*Tg, by cloning full-length *Ctla4* cDNA downstream of the *Il2* promoter and upstream of a CD2 locus control region (LCR) (**Fig II-1A** *Top*). A similar strategy has been used to faithfully report *Il2* gene transcription in a copy number dependent and integration-site independent manner (307). Four Tg founder lines were generated of which two were selected for further studies based on their optimal expression of CTLA-4. These were then backcrossed to *Clta4*$^{-/-}$ mice to generate *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice that were designed to express CTLA-4 only in activated T cells.

To establish the kinetics of Tg*Ctla4* expression in T cells, we crossed the *Il2p-Ctla4*Tg*Ctla4*$^{-/-}$ mice with *5C.C7Tcr*Tg*Rag*$^{-/-}$*Ctla4*$^{-/-}$ mice to obtain a homogenous naïve T cell population. There was no detectable Tg*Ctla4* mRNA expression in *ex vivo* naïve CD4^{+}T cells as shown by RT-PCR (**Fig II-1A** *Bottom*). Upon activation with anti-CD3 and anti-CD28 mAbs, there was rapid up-regulation of Tg*Ctla4*

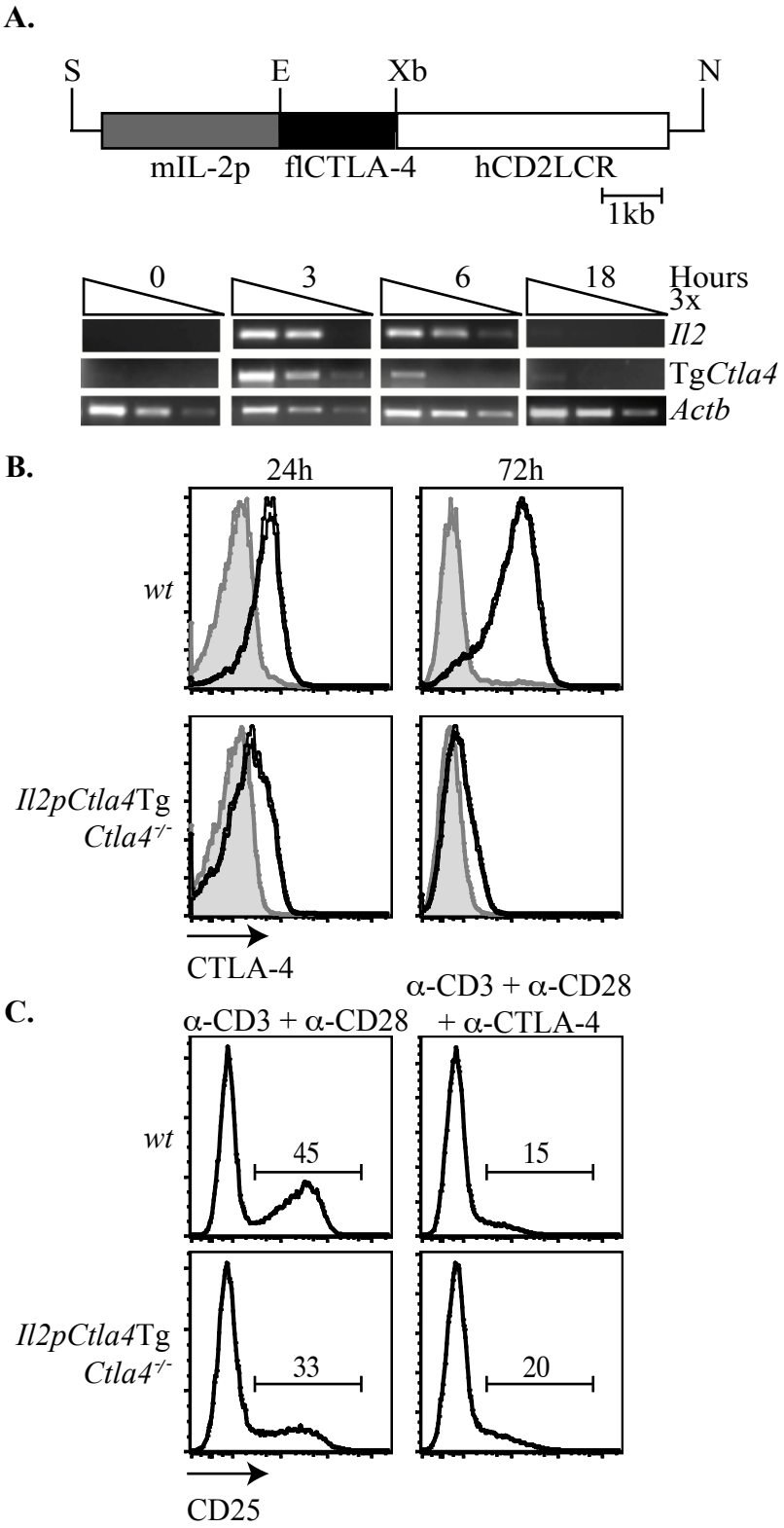

Figure: II-1 Generation of *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice and characterization of Tg function

Figure II-1: Generation of *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice and characterization of Tg function.

A. *(Top)* Restriction enzyme map of *Il2pCtla4*Tg construct. Full length (fl) CTLA-4 cDNA was cloned 3` of the *Il2* promoter and 5` of the human CD2 locus-control region (LCR). Restriction endonuclease sites **S**: Sal1, **E**: EcoR1, **Xb**: Xba1, **N**: Not1. *(Bottom) Il2pCtla4*Tg mice were crossed to *5C.C7Rag*$^{-/-}$*Ctla4*$^{-/-}$ mice. Naïve CD4^{+}T cells from LNs of *5C.C7Il2pCtla4*Tg*Rag*$^{-/-}$*Ctla4*$^{-/-}$ mice were stimulated with plate-bound anti-CD3 plus anti-CD28 Ab for indicated time periods. mRNA was isolated and 3-fold serial dilutions of cDNA were analyzed for expression of *Il2* and *Ctla4* transgene (Tg*Ctla4*) by RT-PCR. Data are representative of two independent experiments. **B.** FACS sorted CD4^{+}CD25^{-} cells from *wt* and *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice were stimulated with either anti-CD3 plus anti-CD28 coated Sepharose beads for 24h and 72h. Total CTLA-4 expression on activated CD25^{+}CD69^{+}CD4^{+} T cells was determined by flow cytometry. *Ctla4*$^{-/-}$ CD4^{+}T cells were used as a negative control for staining (filled histograms). **C.** CD4^{+}CD25^{-} cells from *wt* and *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice were stimulated with either anti-CD3 plus anti-CD28 Ab and control hamster IgG or anti-CD3 plus anti-CD28 and anti-CTLA-4 coated Sepharose beads. Frequency of activated CD4^{+}CD25^{+} cells T cells was determined by flow cytometry at 24 hours post-stimulation. Data in B and C are representative of two independent experiments each.

transcripts within the first 3 hours, which correlated with the kinetics of accumulation of endogenous *Il2* mRNA.

To determine CTLA-4 protein expression, naive $CD4^+CD25^-$ T cells were sorted from 3-4 weeks old *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice and activated *in vitro*. Intracellular (ic) Tg*Ctla4* expression was detected in activated ($CD44^{hi}CD62L^{lo}CD69^+$) $CD4^+$ T cells at 24 hrs that diminished by 72 hrs post-activation (**Fig II-1B**). In contrast, activated *wt* $CD4^+$ T cells accumulated CTLA-4 protein over time. The functionality of Tg*Ctla4* expression was confirmed, as cross-linking CTLA-4 on cell surface using anti-CTLA-4 coated Sepharose beads resulted in comparably decreased activation, as seen by a reduction in the expression of activation marker, CD25, (**Fig II-1C**) on *Il2pCtla4*Tg*Ctla4*$^{-/-}$ and *wt* $CD4^+$T cells.

Tg*Ctla4* expression in activated T cells is insufficient to prevent lymphoproliferation, but sufficient to prevent early onset fatal autoimmunity.

Ctla4$^{-/-}$ mice die by 3 weeks of age due to lymphoproliferation, aggressive multi-organ infiltration by activated T cells and consequent tissue damage (169, 301). *Il2* promoter driven CTLA-4 expression prevented mortality and dramatically extended the life span of *Ctla4*$^{-/-}$ mice as ~50% of mice lived till 10-12 months of age (**Fig. II-2A**).

The survival benefit of Tg*Ctla4* did not come about because peripheral naïve T cells were regulated properly. There was an increased proportion of activated ($CD44^{hi}CD62L^{neg}IL\text{-}7R^{lo}$) $CD4^+$ T cells in peripheral LNs of *Il2pCtla4*Tg*Ctla4*$^{-/-}$

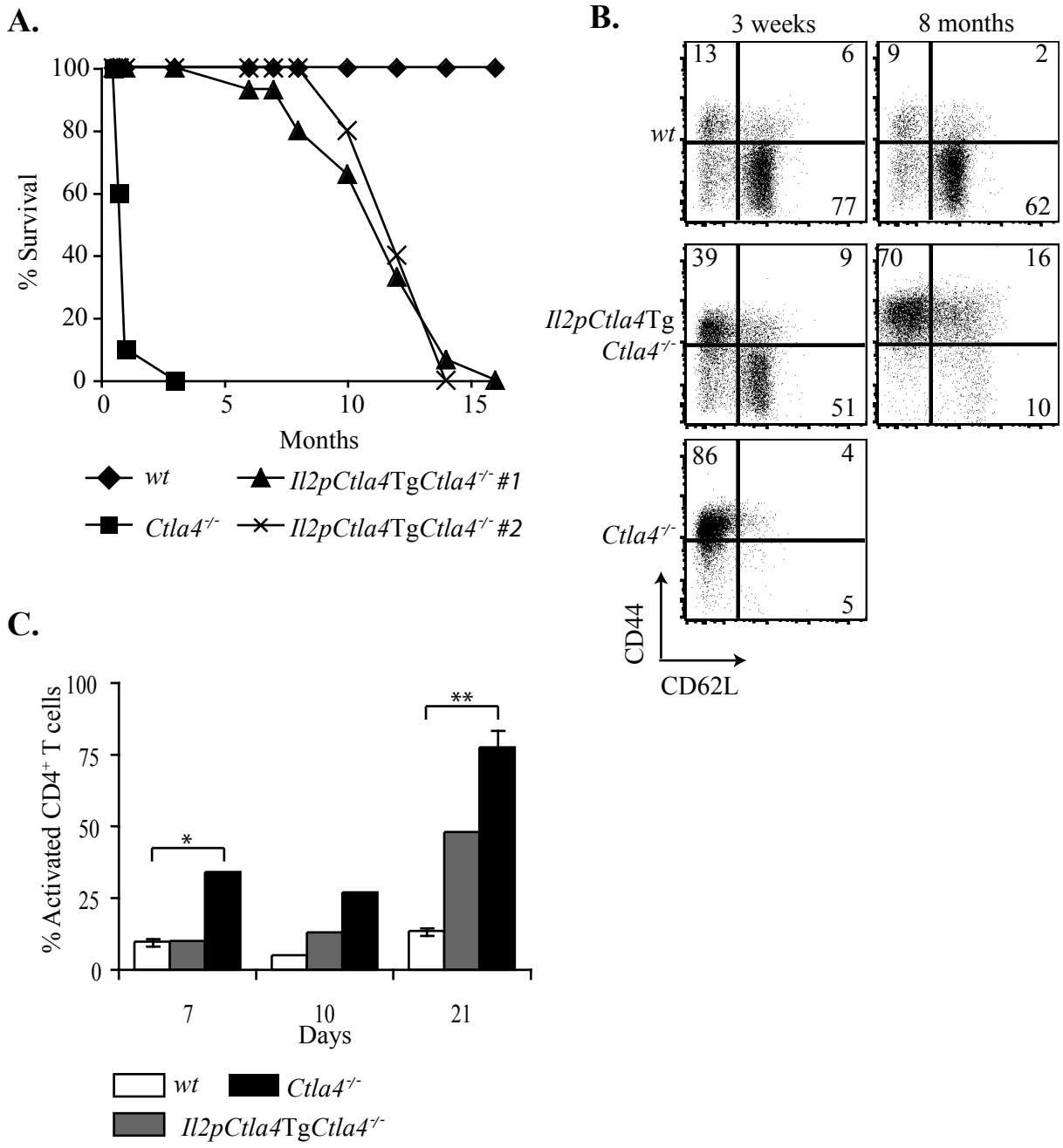

Figure: II-2: Activated T cell restricted expression of CTLA-4 delays lethality but cannot prevent lymphocyte activation

Figure II-2: Activated T cell restricted expression of CTLA-4 delays lethality but cannot prevent lymphoproliferation.

A. *wt* (n=10, ♦), *Ctla4*$^{-/-}$ (n=10, ■) and 2 founder lines of *Il2pCtla4*Tg*Ctla4*$^{-/-}$ (n=15, ▲, ✕) mice were observed for signs of disease and mortality over the indicated months. Results are represented as percent survival, which was calculated as 100 x (number of surviving mice/total number of mice) at each time point. **B.** Representative flow cytometric dot plot showing expression of activation markers CD44 and CD62L on CD4^{+}FOXP3^{-} T cells in peripheral LNs of *wt* and *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice at 3 wks and 8 months of age and *Ctla4*$^{-/-}$ mice at 3 wks of age. Data are representative of at least three independent experiments with 2-3 mice per group. **C.** Frequency of CD4^{+}FOXP3^{-}CD44^{hi}CD62L^{lo} cells in the spleens of 7, 10 and 21 days old *wt, Il2pCtla4*Tg*Ctla4*$^{-/-}$, and *Ctla4*$^{-/-}$ mice. Data are representative of at least three independent experiments with 2-3 mice per group.

(* p=<0.05, ** p=<0.001)

mice at 3 weeks of age, a trend that was detected as early as 10 days after birth (**Fig. II-2B, C**). By 8 months nearly all $CD4^+$ T cells displayed an activated phenotype (**Fig. II-2B**). Further, these cells produced copious amounts of cytokines typical of activated/memory cells, such as IL-2, IFNγ, and TNFα (data not shown). The activated T cells were proliferating, since BrdU labeling experiments at 8 weeks of age showed that there was a 2-3 fold increase in the frequency of $CD4^+$T cells that had incorporated the nucleotide analog in their DNA (**Fig II-3A**), that was mirrored by a significant increase in lymph node (LN) cellularity, starting at ~2-3 months of age (**Fig II-3B**). The expansion of lymphocytes was initially driven by an increase in $CD4^+$T cell numbers (**Fig II-3C**), similar to the pattern in *Ctla4*$^{-/-}$ mice.

To account for the extended survival of *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice compared to *Ctla4*$^{-/-}$ mice in spite of aberrant naive T cell activation, we considered three non-mutually exclusive possibilities. First, while Tg*Ctla4* expression in T cells was globally affording protection against fatal autoimmunity, it was ineffective in preventing aberrant T cell activation due to a combined alteration in the kinetics and levels of CTLA-4 expression. Second, although Treg cells require IL-2 for their development (72, 308) and maintenance (309), they themselves transcribe very little *Il2* (310), and further, FOXP3 represses *Il2* gene transcription (121, 122). Hence, it was predicted that peripheral Treg cells in *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice would not express the Tg*Ctla*4 since IL-2 promoter is actively suppressed in Treg cells. Further, we have recently demonstrated that in mixed chimeras containing *wt* and *Ctla4*$^{-/-}$ T cells, CTLA-4^+ Treg cells inhibit the activation of *Ctla4*$^{-/-}$ T cells in trans and that

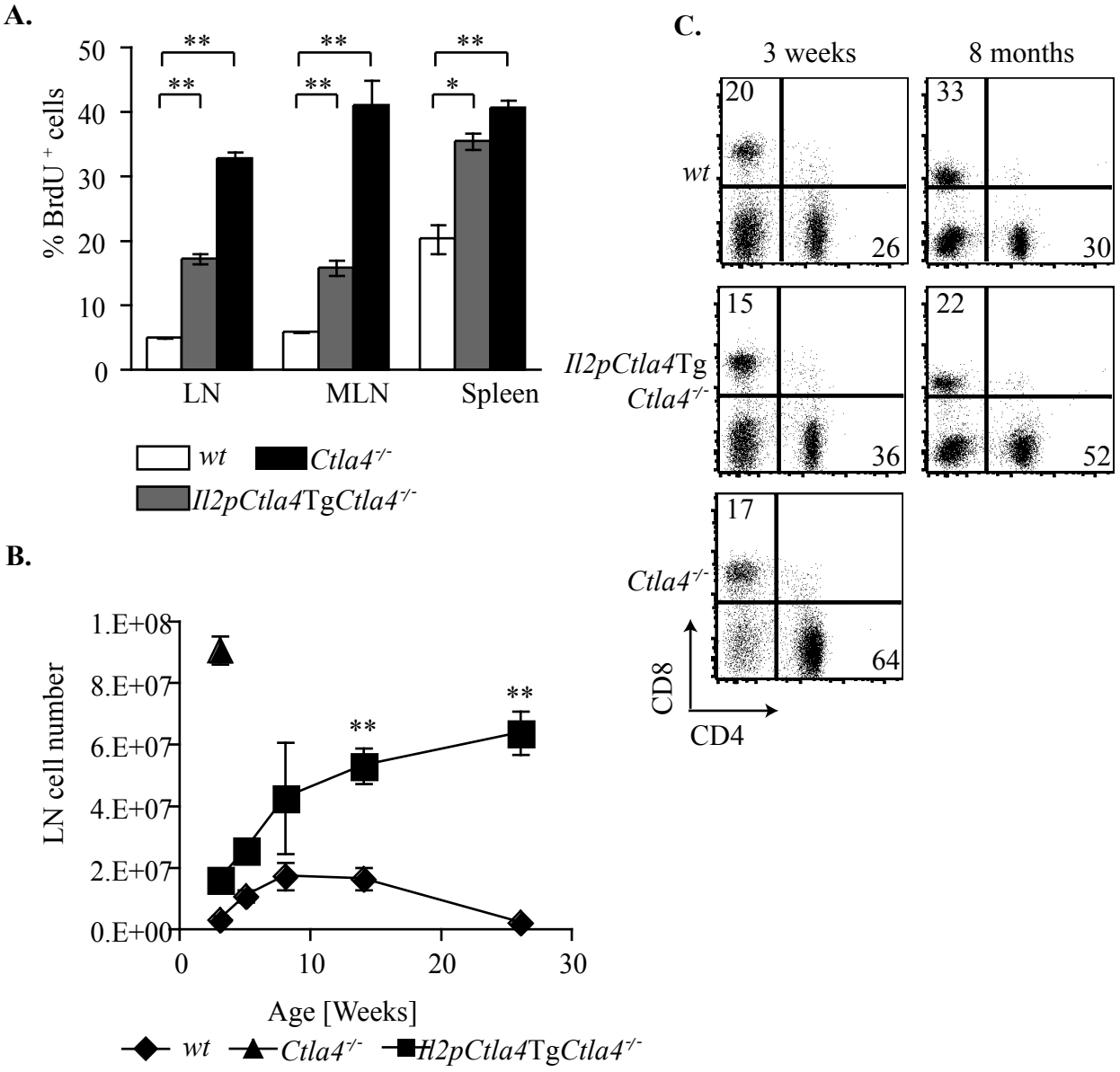

Figure II-3: Activated T cell restricted expression of CTLA-4 cannot prevent lymphoproliferation.

Figure II-3: Activated T cell restricted expression of CTLA-4 cannot prevent lymphoproliferation.

A. *wt* (8-10 wks old), *Ctla4*$^{-/-}$ (3-4 wks old) and *Il2pCtla4*Tg*Ctla4*$^{-/-}$ (8-10 wks old) mice were injected with bromo-deoxyuridine (BrdU) once every 12 hours for 2 days. Frequency of cycling CD4^{+} T cells in lymph nodes (LN), mesenteric lymph nodes (MLN) and spleen was determined by flow cytometric analysis of BrdU incorporation. Data are a mean of four mice per group. **B.** Total cellularity of pooled inguinal, axillary and brachial LNs in *wt* (▲) and *Il2pCtla4*Tg*Ctla4*$^{-/-}$ (■) mice at different ages. Data are a mean of at least four mice per group at each time point. **C.** Representative flow cytometric dot plot showing frequency of CD4 and CD8 T cells in LN, MLN and spleen of *wt* and *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice at 3 wks and 8 months of age and *Ctla4*$^{-/-}$ mice at 3 wks of age. Data are representative of at least five independent experiments with 2-3 mice per group.

(* p=<0.05, ** p=<0.001)

normally, Treg cells may dominantly maintain the quiescence of naïve T cells (Friedline et al., *J. Exp. Med.* in press). Therefore, lack of CTLA-4 in the Treg cells was causing the aberrant naïve T cell activation in *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice, while the restricted Tg*Ctla4* expression in activated T cells was inhibiting the disease progression directly. Third, it was possible that the Treg cells express low levels of CTLA-4 that are insufficient to regulate naïve T cell activation, but capable of moderating the self-destructive activities of aberrantly activated T cells incapable of expressing CTLA-4 normally.

FOXP3^{+}Treg cells from *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice are functionally impaired

To distinguish between these possibilities we first examined CTLA-4 expression in T cell subsets of *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice and tested whether Treg cells in the mice are functional. In the thymus of *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice, Tg*Ctla4* expression pattern was similar to that of endogenous CTLA-4. Tg*Ctla4* was expressed in the early αβ T cell lineage precursor cells, CD4^{-}CD8^{-}CD44^{-}CD25^{+} (DN3), and the level of Tg expression was comparable to *wt* thymic precursors (**Fig. II-4A**). However, Tg*Ctla4* was not expressed significantly in all other thymocyte subsets, except in CD4^{+}SP FOXP3^{+} thymocytes where intracellular (ic) Tg*Ctla4* was detected (**Fig. II-4B**) (see Discussion). In the periphery, CTLA-4 expression could be detected only in activated CD4^{+}CD44^{hi} T cells (**Fig. II-4C**). Importantly, FOXP3^{+} Treg cells did not express high amounts of ic CTLA-4 as compared to those from *wt* mice and no, or trace, cell surface CTLA-4 expression was observed on Treg cells (**Fig. II-5C**). In summary, the expression pattern of CTLA-4 in *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice indicated that the Tg was being regulated *in vitro* and *in vivo* as designed, since functional

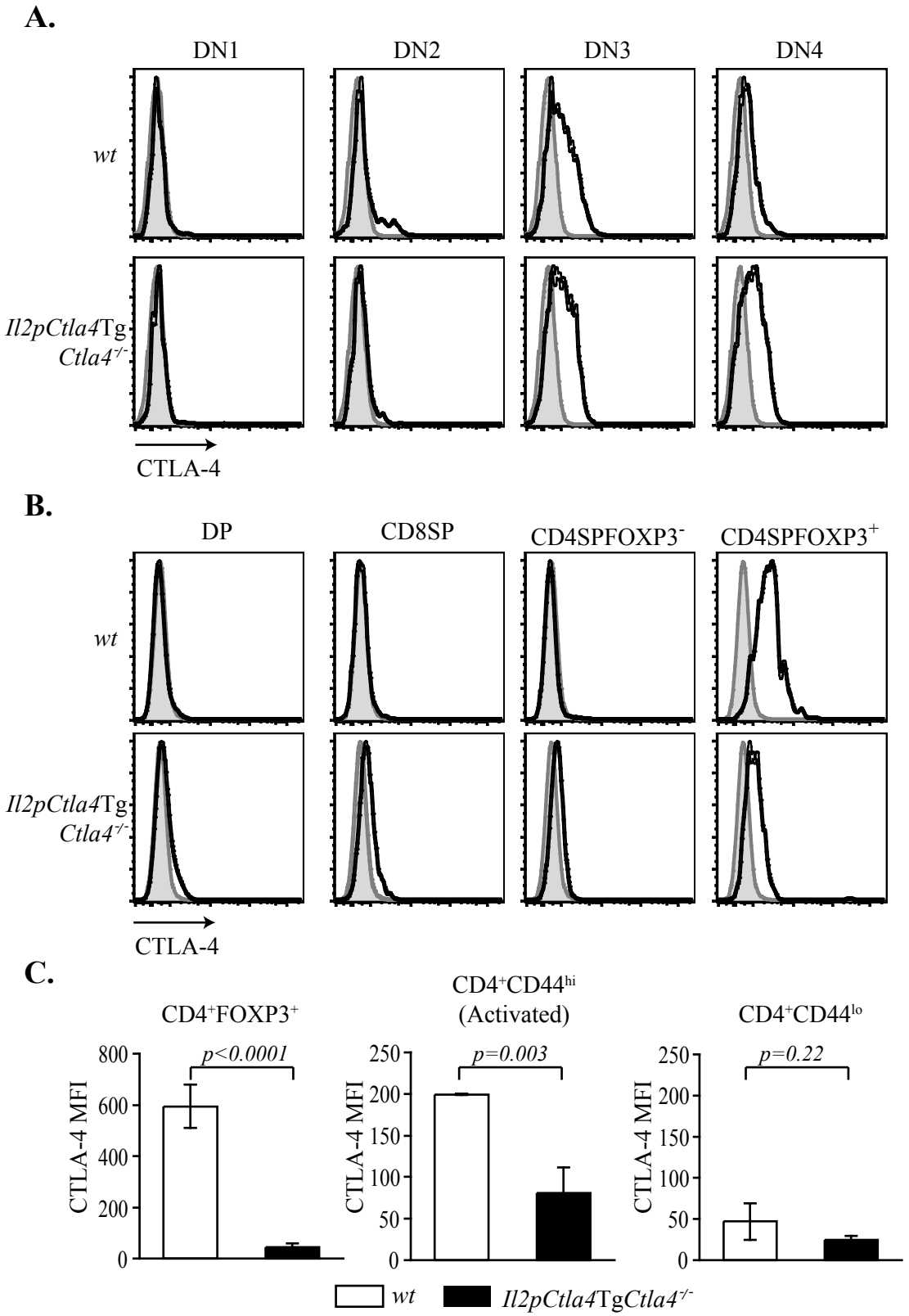

Figure II-4: Expression of Tg*Ctla4* in T cell subsets of *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice.

Figure II-4: Expression of Tg*Ctla4* in T cell subsets of *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice.

A, B. Thymocytes from *wt* (6-12wks old), *Ctla4*$^{-/-}$ (3-4wks old) and *Il2pCtla4*Tg*Ctla4*$^{-/-}$ (6-12wks old) mice were analyzed for expression of intra-cellular CTLA-4 by flow cytometry. **DN1**: $CD4^-CD8^-CD44^+CD25^-$; **DN2**: $CD4^-CD8^-CD44^+CD25^+$; **DN3**: $CD4^-CD8^-CD44^-CD25^+$; **DN4**: $CD4^-CD8^-CD44^-CD25^-$; **DP**: $CD4^+CD8^+$; **CD8SP**: $CD4^-CD8^+$; **CD4SP**: $CD4^+CD8^-$. *Ctla4*$^{-/-}$ thymocytes were used as a negative control for CTLA-4 expression (filled histogram). Data are representative of three independent experiments. **C.** CTLA-4 expression in peripheral T cell subsets from 6-12 wks old *wt* and *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice was determined by flow cytometry. Data are the average of Mean Fluorescence Intensity (MFI) of CTLA-4 expression determined over at least five independent experiments with 2-3 mice in each group per experiment. For comparison, background CTLA-4 staining from *Ctla4*$^{-/-}$ $CD4^+$T cells had MFI of 30. *p*-values indicated on graph.

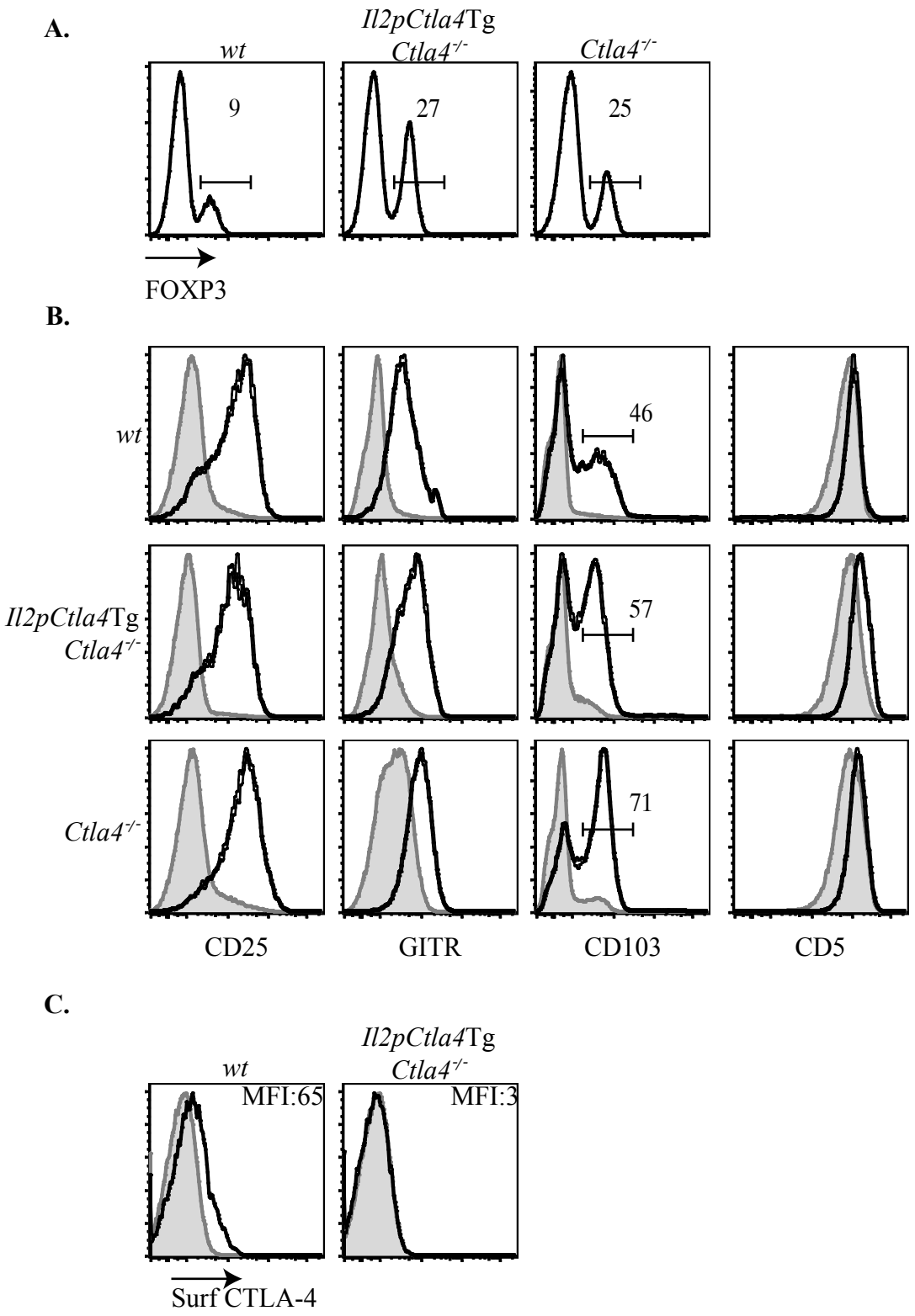

Figure II-5: Characterization of Treg cells from *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice.

Figure II-5: Characterization of Treg cells from *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice.

A. Frequency of CD4^{+}FOXP3^{+} Treg cells in *wt* (6-12 wks old), *Ctla4*$^{-/-}$ (3-4 wks old) and *Il2pCtla4*Tg*Ctla4*$^{-/-}$ (6-12 wks old) mice was determined by flow cytometry. Data are representative of at least five independent experiments with 3-4 mice in each group. **B.** Total LN cells from *wt*, *Ctla4*$^{-/-}$ and *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice were analyzed for the expression of FOXP3 by flow cytometry. FOXP3^{+} (unfilled histogram) and FOXP3^{-} (filled histogram) cells were further compared for the expression of CD25, GITR, CD103 and CD5. Data are representative of at least five independent experiments with 3-4 mice in each group. **C.** CD4^{+}FOXP3^{+} T cells from *wt* (*Left*) and *Il2pCtla4*Tg*Ctla4*$^{-/-}$ (*Right*) mice were analyzed for expression of surface CTLA-4. *Ctla4*$^{-/-}$ CD4^{+} T cells were used as a negative control for CTLA-4 expression (gray histogram). Numbers in FACS plots are the mean fluorescence intensity of CTLA-4 staining normalized to *Ctla4*$^{-/-}$ control.

CTLA-4 is expressed only in activated T cells in the periphery and not significantly in naive T cells and Treg cells.

To determine whether Treg cells from *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice are similar to those of *Ctla4*$^{-/-}$ mice we compared the developmental properties and function of Treg cells from these mice in detail. During development, there is an increased frequency of CD4^{+}FOXP3^{+} T cells in *Ctla4*$^{-/-}$ mice, and there was also a similar 2-fold increase in the frequency of CD4^{+}FOXP3^{+} cells in *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice (**Fig. II-5A**). This increase occurs as early as 7 days after birth in *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice, before overt CD4^{+} T cell activation (data not shown), indicating that it is unlikely a result of increased availability of growth factors like IL-2 in the milieu that enhance Treg cell production and maintenance. Phenotypically, Treg cells from *Il2pCtla4*Tg*Ctla4*$^{-/-}$ and *Ctla4*$^{-/-}$ mice were similar, except for CTLA-4 expression (**Fig. II-5B**).

CD4^{+}CD25^{+} T cells from *Ctla4*$^{-/-}$ mice can suppress the proliferation of responder T cells *in vitro*. Similarly, Treg cells from *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice were also functional *in vitro* (data not shown), albeit to a lesser degree compared to *wt* Treg cells (**Fig. II-6A**). *In vivo*, however, Treg cells from *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice were defective. In an adoptive T cell transfer model of colitis, Treg cells from *Il2pCtla4*Tg*Ctla4*$^{-/-}$ or *Ctla4*$^{-/-}$ mice were not able to prevent colitis initiated by co-injected *wt* naïve T cells. While *wt* Treg cells were able to efficiently regulate the colitogenic T cells and this cohort of T cell transferred hosts remained healthy (**Fig. II-6B**), the mice receiving *Il2pCtla4*Tg*Ctla4*$^{-/-}$ Treg cells succumbed to a wasting disease, displaying massive lymphocyte infiltration and significant epithelial

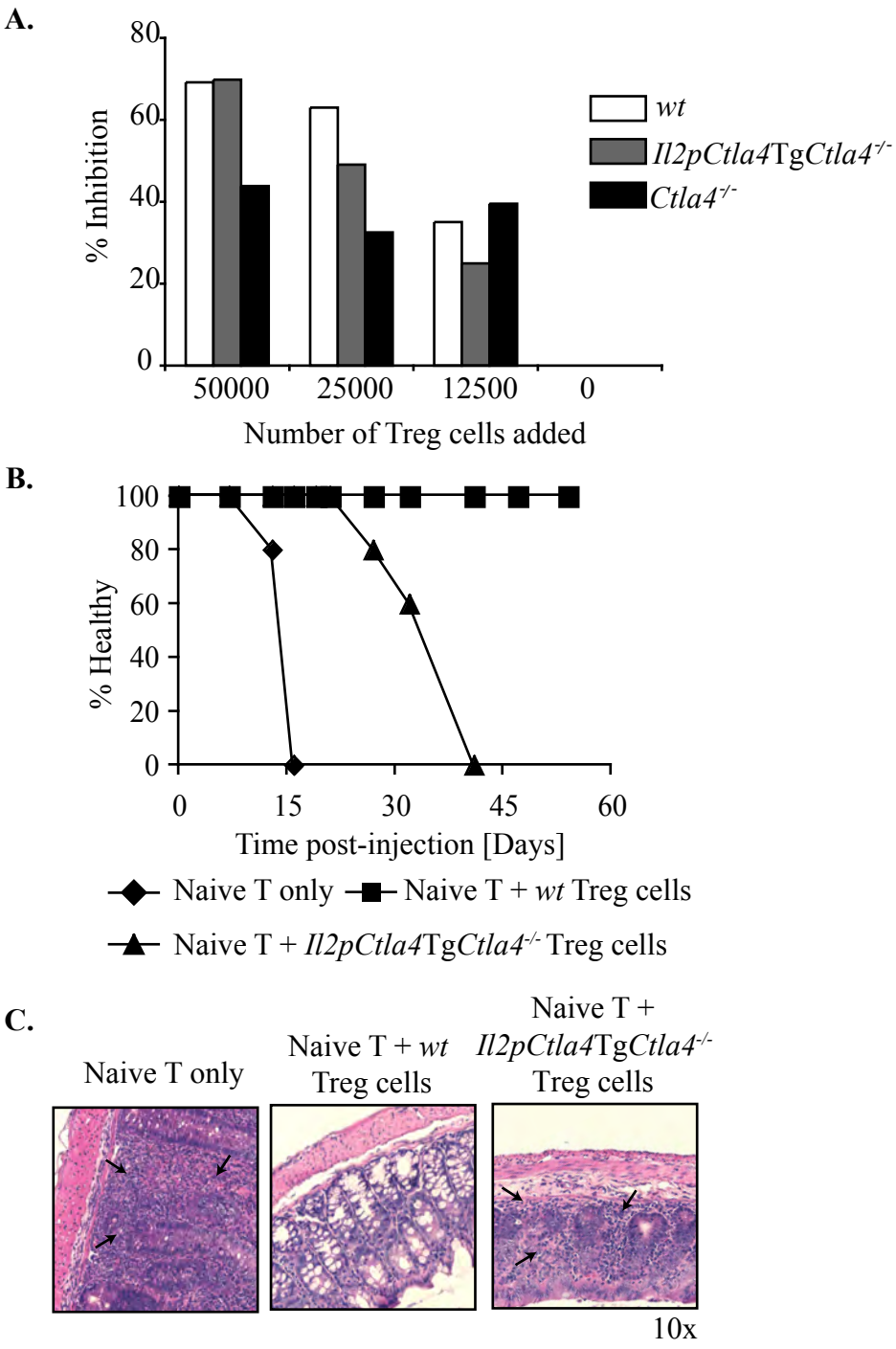

Figure II-6: Treg cells from *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice are functionally impaired i*n vitro* and *in vivo.*

Figure II-6: Treg cells from *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice are functionally impaired.

A. Responder CD4^{+} effector T cells were co-cultured with varying numbers of sorted and pre-activated CD4^{+}CD25^{+} cells from *wt, Il2pCtla4*Tg*Ctla4*$^{-/-}$ and *Ctla4*$^{-/-}$ mice. Cells were stimulated with plate bound anti-CD3 plus anti-CD28 and proliferation was measured by ^{3}H thymidine incorporation at 65 hrs post-activation. % inhibition of proliferation was calculated by considering the proliferation of responder T cells in the absence of Treg cells as 100%. One of 3 independent experiments with similar results is shown. **B, C.** CD4^{+}CD25^{+} Treg cells from *wt*, and *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice were transferred with *wt* CD4^{+}CD25^{neg} T cells into *Rag1*$^{-/-}$ mice to test their ability to prevent colitis. **B**. Mice were scored as "unhealthy" when first signs of illness including weight loss and mucosal inflammation around the eyes were observed. Frequency of healthy mice was plotted and data are representative of two independent experiments with 4-5 mice in each group. *Rag1*$^{-/-}$ mice receiving *wt* CD4^{+}CD25^{-} T cells alone were used as a control for colitis induction. ♦: Naïve T cell only, ■: Naïve T plus *wt* Treg cells, ▲: Naïve T plus *Il2pCtla4*Tg*Ctla4*$^{-/-}$ Treg cells. **C.** Representative H&E stained sections of colons of *Rag1*$^{-/-}$ after transfer of T cells. Mice that received naïve *wt* T cells (*Left panel*) developed severe colitis, which was prevented by co-transfer of *wt* Treg cells (*Middle panel*). Mice constituted with naïve *wt* T cells and *Il2pCtla4*Tg*Ctla4*$^{-/-}$ Treg cells (*Right panel*) developed colitis, displaying substantial inflammatory infiltrates and significant epithelial hyperplasia with loss of goblet cells. However, the severity of colitis was milder as compared to mice receiving only naive *wt* T cells. Original magnification: 10X.

hyperplasia with the loss of mucin secreting goblet cells in the colon (**Fig. II-6C**). This lack of regulatory activity was evident even when a 2-fold higher ratio of Treg:Teff was tested, indicating that the defect was not simply a matter of potency of suppression (data not shown). However, the progression of disease was delayed somewhat, compared to control mice receiving *wt* naïve T cells alone, suggesting that the CTLA-4-less Treg cells may have some residual function.

One caveat of the colitis assay was the possibility that the sorted Treg cells ($CD25^+CD4^+$) from CTLA-4-deficient mice may contain contaminating activated conventional $CD4^+$ T cells that may accelerate the disease progression. There also exist conflicting results regarding the function of CTLA-4-deficient Treg cells in the colitis model. For these reasons, the colitis assay by itself may not accurately report the activities of Treg cells. To alleviate these concerns and to definitively test Treg cell function, we used the mixed BM chimera assay of trans regulation of *Ctla4*$^{-/-}$ T cells. In mice reconstituted with a mixture of *wt* and *Ctla4*$^{-/-}$ BM-derived cells, *Ctla4*$^{-/-}$ T cells are maintained in a normal un-activated state and *wt* Treg cells are necessary and sufficient for this regulation (Friedline et al. *J. Exp. Med.* in press). Hosts reconstituted with BM cells from *Ctla4*$^{-/-}$ or *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice alone are replete with activated ($CD44^+$), proliferating T cells, with multi-organ tissue infiltration by activated T cells in the former, but not the latter (**Fig. II-7**, *Top left* and data not shown).

To determine the functional competence of CTLA-4-less Treg cells in this model, T cell-depleted *wt* and congenically marked *Il2pCtla4*Tg*Ctla4*$^{-/-}$ BM cells

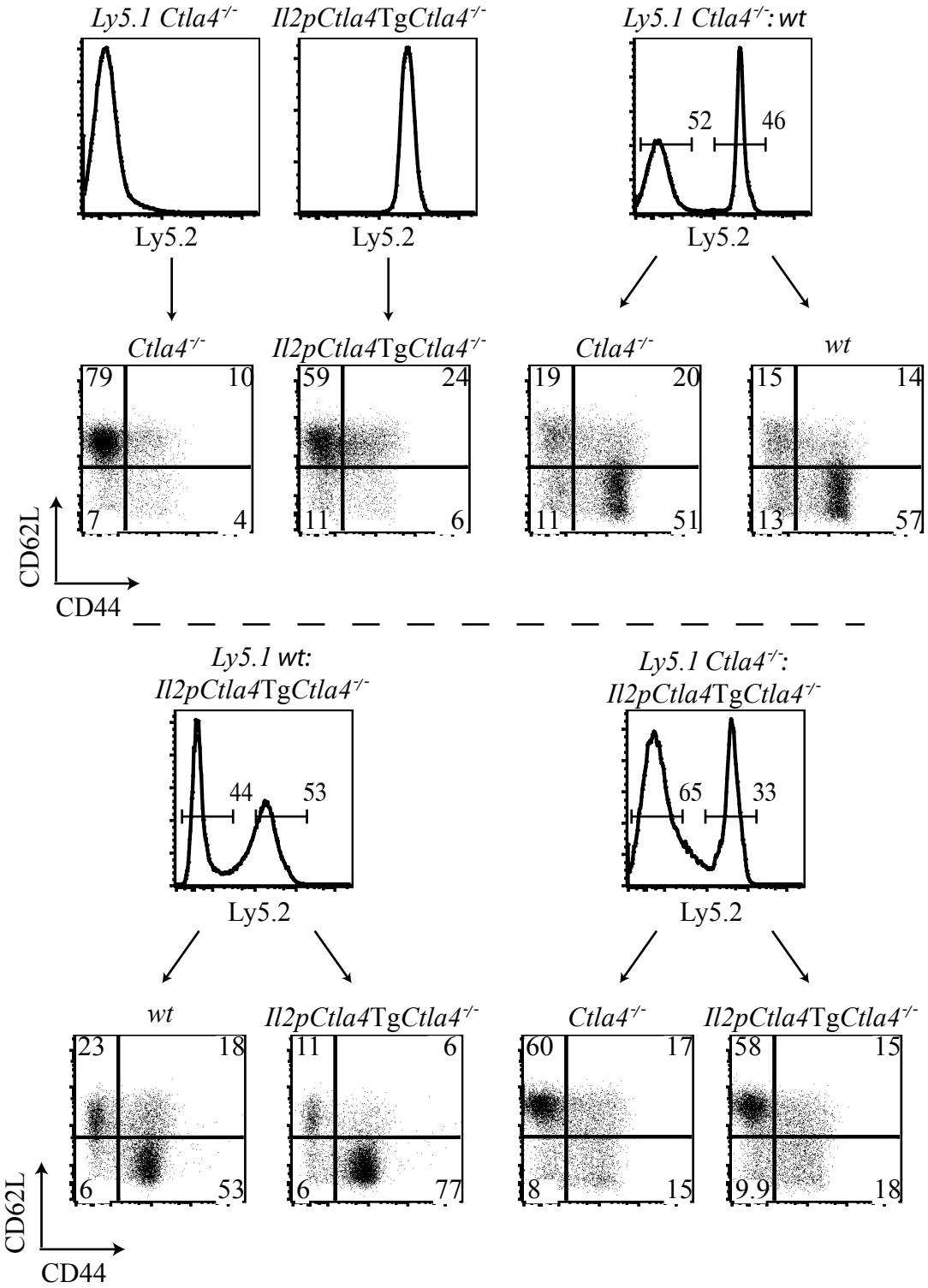

Figure II-7: Treg cells from *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice cannot prevent activation of *Ctla4*$^{-/-}$ lymphocytes in mixed bone marrow chimeras.

Figure II-7: Treg cells from *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice cannot regulate *Ctla4*$^{-/-}$ T cells in mixed BM chimeras (BMCs).

BMCs were generated in *Rag1*$^{-/-}$ mice by injecting T cell-depleted BM cells from either Ly5.1^{+}*Ctla4*$^{-/-}$ or *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice (*Top left, negative controls*), or co-injecting a mix of BM cells from *wt* and Ly5.1^{+}*Ctla4*$^{-/-}$ mice (*Top right, positive control*), Ly5.1 *wt* and *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice (*Bottom left*) and Ly5.1 *Ctla4*$^{-/-}$ and *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice (*Bottom right*). 6-8 weeks after reconstitution, before some BMCs became fatally sick, mice were euthanized and LN cells were stained with Ly5 congenic marker to determine contributions of each group to the peripheral lymphocyte compartment. Ly5.2^{+} and Ly5.2^{-} CD4^{+} T cells were further analyzed for the expression of activation markers, CD44 and CD62L. Data are representative of two independent experiments with three mice in each group.

were mixed at a 1:1 ratio and injected into sub-lethally irradiated *Rag1*$^{-/-}$ mice. In the presence of *wt* BM-derived T cells (~1:1 wth test T cells), *Ctla4*$^{-/-}$ or *Il2pCtla4*Tg*Ctla4*$^{-/-}$ T cells did not expand and remained mostly naïve (CD44$^{\mathbf{lo}}$), similar to *wt* T cells in the same mouse (**Fig. II-7**, *Top right* and *bottom left*). The result indicated that the aberrant T cell activation in *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice occurred because the Treg cells are defective in maintaining naïve T cell homeostasis.

To directly test whether CTLA-4-less Treg cells from *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice still retained the ability to prevent aberrantly activated T cells from infiltrating tissues, *Il2pCtla4*Tg*Ctla4*$^{-/-}$: *Ctla4*$^{-/-}$ mixed chimeras were analyzed. *Il2pCtla4*Tg*Ctla4*$^{-/-}$ Treg cells failed to regulate the activation and expansion of *Ctla4*$^{-/-}$ CD4^{+} T cells, as expected, and more tellingly, the chimeras rapidly succumbed to fatal lymphoproliferative disease with extensive T cell infiltration into vital organs (**Fig. II-7**, *Bottom right* and data not shown). These results demonstrated that Treg cells from *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice cannot prevent activation of *Ctla4*$^{-/-}$ T cells, and furthermore, they cannot stop aberrantly activated T cells from accumulating in non-lymphoid organs.

CTLA-4 expressing aberrantly activated T cells do not accumulate in most non-lymphoid tissues

So far, we have shown that Treg cells from *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice express very low levels of CTLA-4, they are phenotypically indistinguishable from *Ctla4*$^{-/-}$ Treg cells, and are functionally impaired *in vivo*. Conversely, provision of *wt* Treg cells can completely prevent aberrant activation of *Il2pCtla4*Tg*Ctla4*$^{-/-}$ T cells. These

results confirm that CTLA-4 is necessary for naïve T cell homeostasis and that the impaired peripheral Treg cells are unlikely to prolong the lifespan of *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice. Instead, while Tg*Ctla4* expression in Tconv cells (**Fig. II-4C**) is not the primary determinant of the activation state of naïve T cells, it appears capable of regulating aberrantly activated T cell pathogenesis.

To determine how activated Tconv cell-intrinsic expression of CTLA-4 controls T cell homeostasis we first investigated why *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice do not succumb to early onset fatal autoimmunity. Analysis of cell surface expression of the apoptosis marker Annexin V showed that T cells were not increased in susceptibility to apoptosis (data not shown). Instead, in stark contrast to *Ctla4*$^{-/-}$ mice Tg*Ctla4* expression was preventing activated T cells from infiltrating into and/or persisting in non-lymphoid organs, as most tissues examined did not show extensive lymphocyte accumulation, even at 6-8 months of age (**Fig. II-8**).

As the mice aged there were two tissues where Tg*Ctla4* expression did not provide protection: the small intestine and pancreas. In both tissues, there was extensive mononuclear cell infiltration, and in the pancreas, there were evidence of T cell infiltration of β-islet cells in older *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice (**Fig. II-9**). However, the mice were not hyperglycemic up to ~10 months of age (data not shown). The small intestine, but not the colon, showed diffused hyperplasia, increased plasma B cell and necrotic cell numbers in the mucosal epithelia, but limited epithelial cell damage (**Fig. II-9B**). Further, there was an increased $CD4^+$ T cell frequency in the intestines and Peyer's patches of old *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice (**Fig. II-10A**).

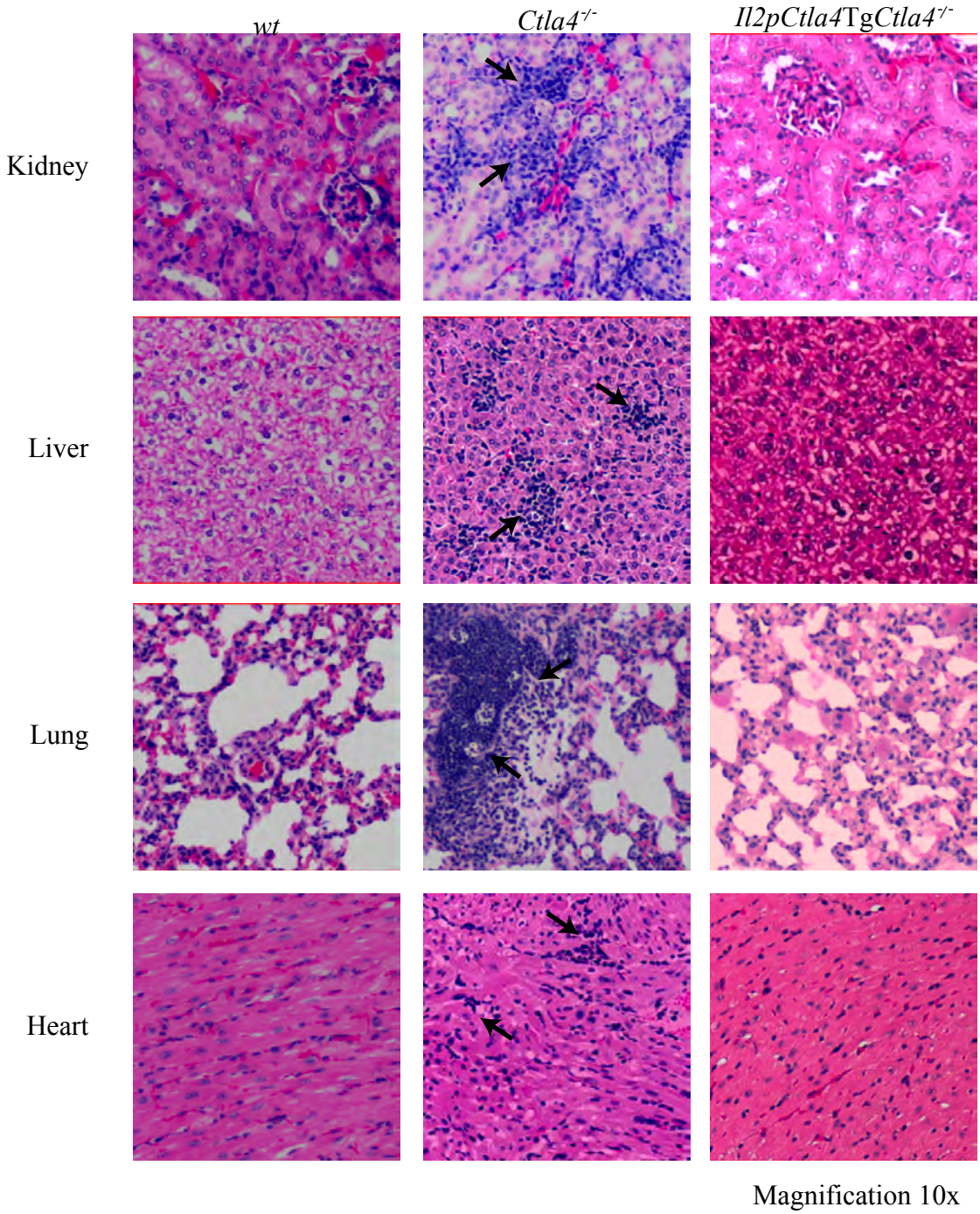

Figure II-8: Lack of non-lymphoid lymphocyte infiltration in *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice

Figure II-8: Lack of non-lymphoid organ specific lymphocyte infiltration in *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice.

Lymphocyte infiltration in *wt* (7 months old), *Ctla4*$^{-/-}$ (3-4 wks old) and *Il2pCtla4*Tg*Ctla4*$^{-/-}$ (7 months old) mice was determined by histology. Representative H&E stained sections of kidney, liver, lung and heart showed extensive lymphocyte infiltration only in sick *Ctla4*$^{-/-}$ mice. Original magnification: 10X.

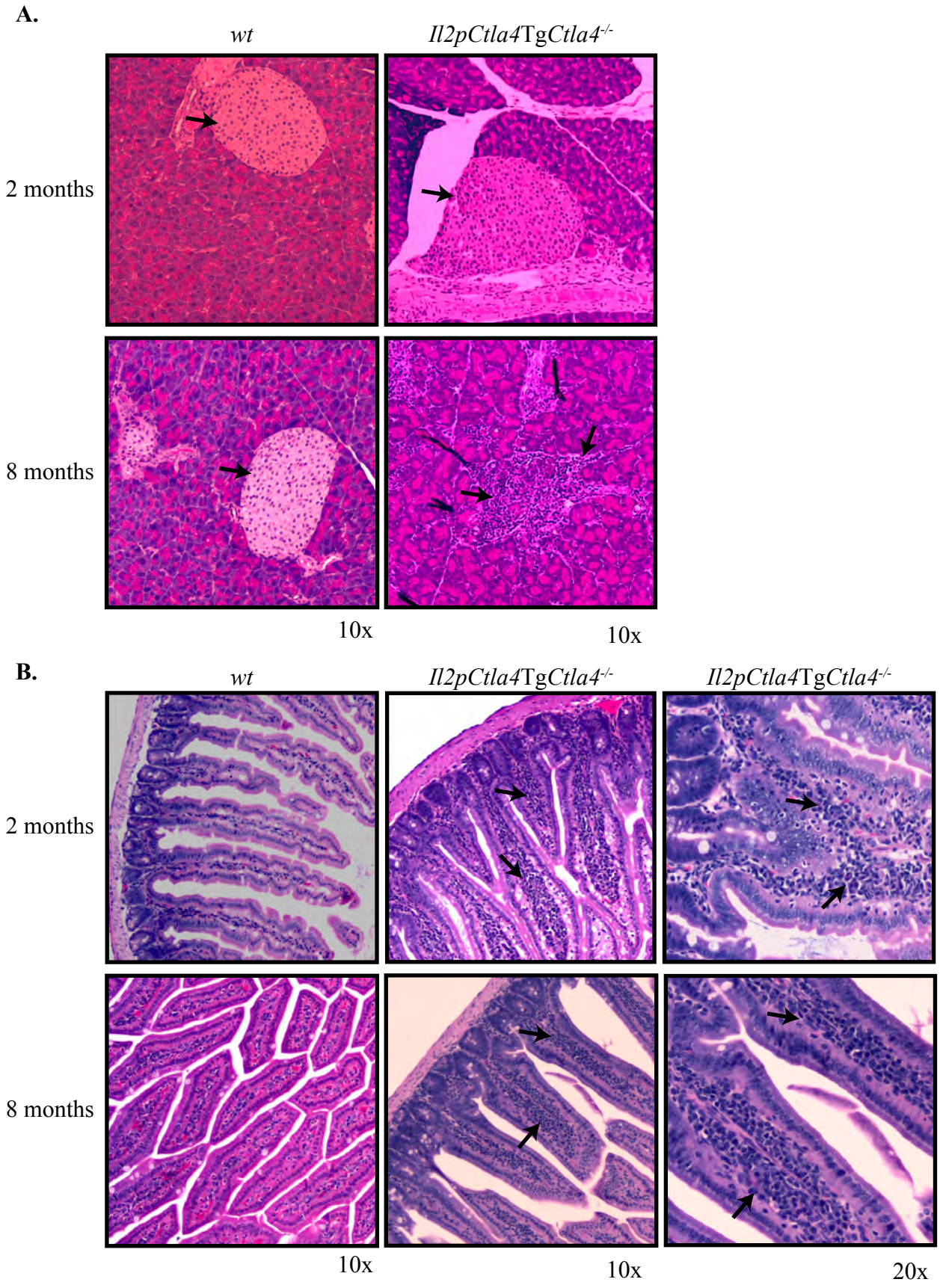

Figure II-9: Organ-specific autoimmunity in *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice.

Figure II-9: Organ specific lymphocyte infiltration in *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice.

Lymphocyte infiltration in *wt* (7 months old), *Ctla4*$^{-/-}$ (3-4 wks old) and *Il2pCtla4*Tg*Ctla4*$^{-/-}$ (7 months old) mice was determined by histology. **A.** Pancreatic β-islets and **B.** small intestines were infiltrated with lymphocytes in old *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice. At least two sections/mouse and multiple fields were analyzed. Original magnification: 10X and 20x (for B)

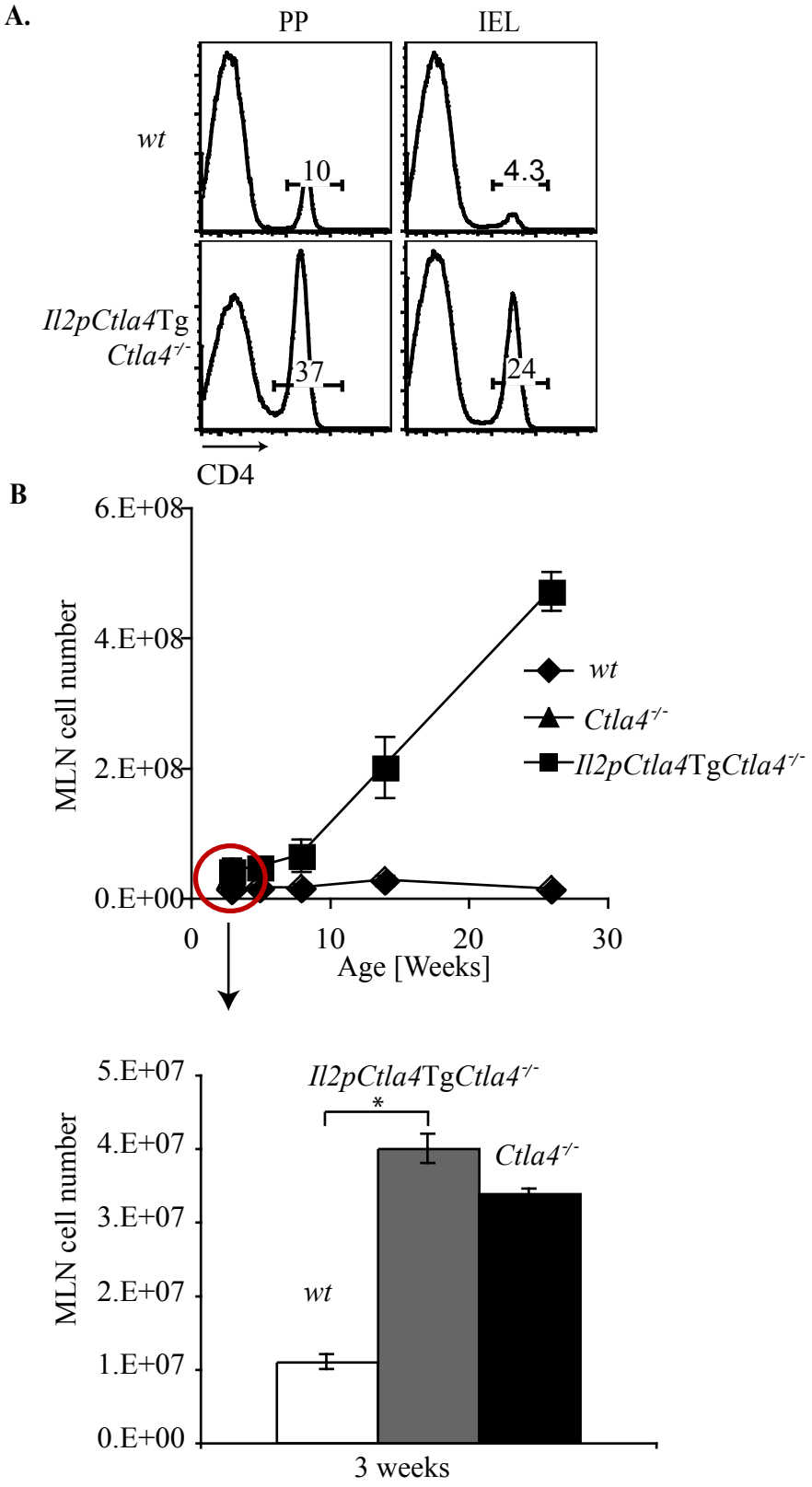

Figure II-10: Altered intestinal homeostasis in *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice.

Figure II-10: Altered intestinal homeostasis in *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice.

A. Representative flow cytometric data showing frequency of CD4^{+}T cells in Peyer's Patches (PP) and intestinal intra-epithelial compartment (iIEL). Data are a mean on three independent experiments with 2-3 mice in each group. **B.** Total cellularity of MLNs at different ages in *wt* (◆), *Ctla4*$^{-/-}$ (▲) and *Il2pCtla4*Tg*Ctla4*$^{-/-}$ (■) mice. Data are a mean of at least four mice per group at each time point. *Ctla4*$^{-/-}$ mice had MLN cellularity of $3.4x10^{7}$ cells; S.D. 650,000. Bar graph depicts the MLN cellularity of 3 weeks old *wt*, *Ctla4*$^{-/-}$ and *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice.

(* p=<0.05)

Accompanying the inflammation was a massive increase in the size of mesenteric LNs of 6-7 months old *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice (**Fig. II-10B**). The gut-associated inflammation in *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice may be exacerbated by the decreased Tg*Ctla4* expression in activated T cells in the small intestine (data not shown). It is likely that the enteritis leads to death of *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice since they can be maintained in good health beyond 10 months when fed antibiotic water (data not shown) that moderates the gut inflammation, and there are no significant infiltration of T cells into other tissues, even in *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice with full-blown enteritis.

To begin to address the homing characteristics of *Il2pCtla4*Tg*Ctla4*$^{-/-}$ CD4^{+}T cells, we examined integrin and chemokine receptor gene expression. There was increased expression of the gut-homing receptor CD103 on activated CD4^{+}T cells from secondary lymphoid organs of *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice (**Fig. II-11A**). Further, while there was no change in the gut and pancreas homing receptor $\alpha4\beta7$ integrin expression on LN cells, there was a marked increased frequency of cells expressing this receptor in the spleen, MLN and IELs of *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice **(Fig II-11B**). Strikingly, the expression of LN homing/retention receptor CCR7 that normally becomes downregulated during activation was maintained in the majority of CD44^{+} *Il2pCtla4*Tg*Ctla4*$^{-/-}$ T cells (**Fig. II-11C**). In contrast, activated *Ctla4*$^{-/-}$ T cells had downregulated CCR7 expression. These results indicate that one aspect of T cell activation that CTLA-4 regulates in a T cell-intrinsic manner is the modifications of aberrantly activated T cell trafficking to non-lymphoid organs.

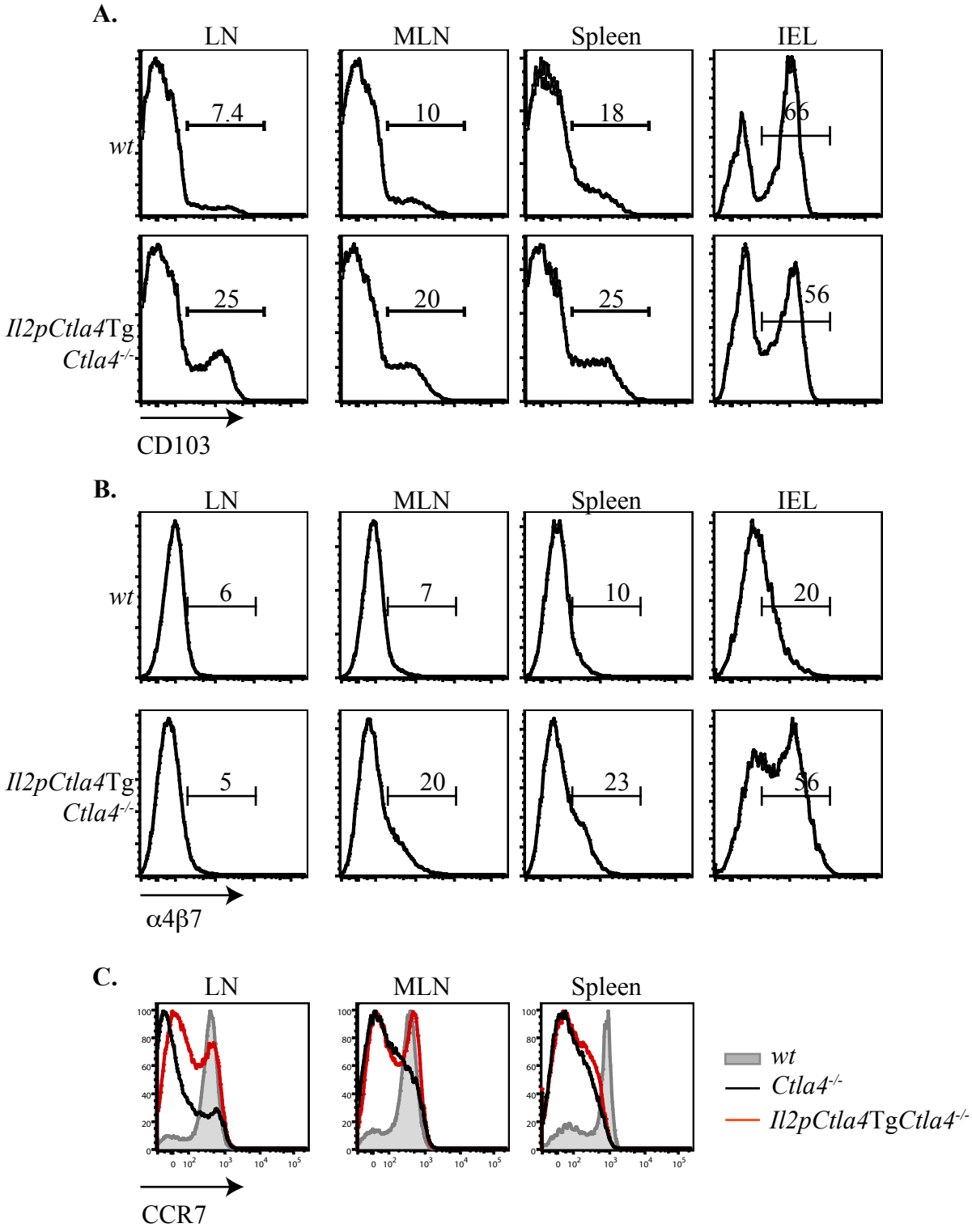

Figure: II-11: Altered expression of homing receptors on CD4⁺ T cells in *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice

Figure II-11: Altered expression of homing receptors on $CD4^+$T cells in *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice.

A, B. Expression of gut homing receptors CD103 (**A**) and α4β7 (**B**) on $CD4^+CD44^{hi}$ activated T cells from peripheral LNs, MLN, spleen and iIELs of 2-3 months old *wt* and *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice as determined by flow cytometry. Data are representative of three independent experiments with 3-4 mice in each group. **(C)** Expression on CCR7 on $CD4^+CD25^-CD44^+$ cells from peripheral LNs, MLN, and spleen of 6-8 months old *wt* and *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice and 3 weeks old *Ctla4*$^{-/-}$ mice as determined by flow cytometry. (Filled gray histogram: *wt*; Black histogram: *Ctla4*$^{-/-}$; Red histogram: *Il2pCtla4*Tg*Ctla4*$^{-/-}$). Data are representative of two independent experiments with 2-3 mice in each group.

OX40-Cre mediated deletion of *Ctla4* in FOXP3$^+$ T cells followed by the loss of CTLA-4 in activated T cells results in fatal autoimmunity

To confirm that CTLA-4 expression in conventional activated T cells is necessary for preventing autoimmune tissue infiltration, we generated a mouse model amenable to conditional, sequential loss of CTLA-4 in FOXP3$^+$ Treg cells, followed by loss in activated T cells. We generated mice in which the *Cd2* promoter drives the expression of a "floxed" full-length *Ctla4* Tg (*flCtla4*Tg; **Fig. II-12A**) in all T cells. *flCtla4*Tg mice were crossed to *Ctla4*$^{-/-}$ mice so that *Ctla4* expression can be conditionally ablated. Interestingly, mice that were heterozygous for *flCtla4*Tg expression (**Fig. II-12B**) could not be maintained in a healthy state for more than 2 months and had increased frequency of activated T cells in peripheral lymphoid organs (**Fig. II-12C**). However, *flCtla4*Tg$^{+/+}$*Ctla4*$^{-/-}$ were healthy with a normal lifespan, suggesting that high CTLA-4 expression was required to maintain *Ctla4*$^{-/-}$ T cells in a naïve state.

To sequentially ablate *Ctla4* in T cell subsets we employed OX40-Cre mice (311). OX40 is a late activation marker that is constitutively expressed on FOXP3$^+$ Treg cells and is induced on conventional T cells by 36-48 hours post-activation (312). Expression of CRE from the OX40 promoter resulted in efficient deletion of the *flCtla4*Tg in nearly all CD4$^+$FOXP3$^+$ T cells by 7-10 days after birth (**Fig. II-13A;** *Top row*). As conventional T cells become activated by the loss of CTLA-4 on Treg cells (**Fig. II-13B)** they also lose CTLA-4 with slower kinetics, starting ~10 days of age, with complete loss of CTLA-4 expression by 2-3 weeks of age (**Fig. II-13A;** *Middle row*). Nearly half of CD4$^+$FOXP3$^-$CD44^{lo} T cells from *Ox40*CreTg$^+$*flCtla4*Tg$^+$*Ctla4*$^{-/-}$ mice maintained the expression of CTLA-4 at 10

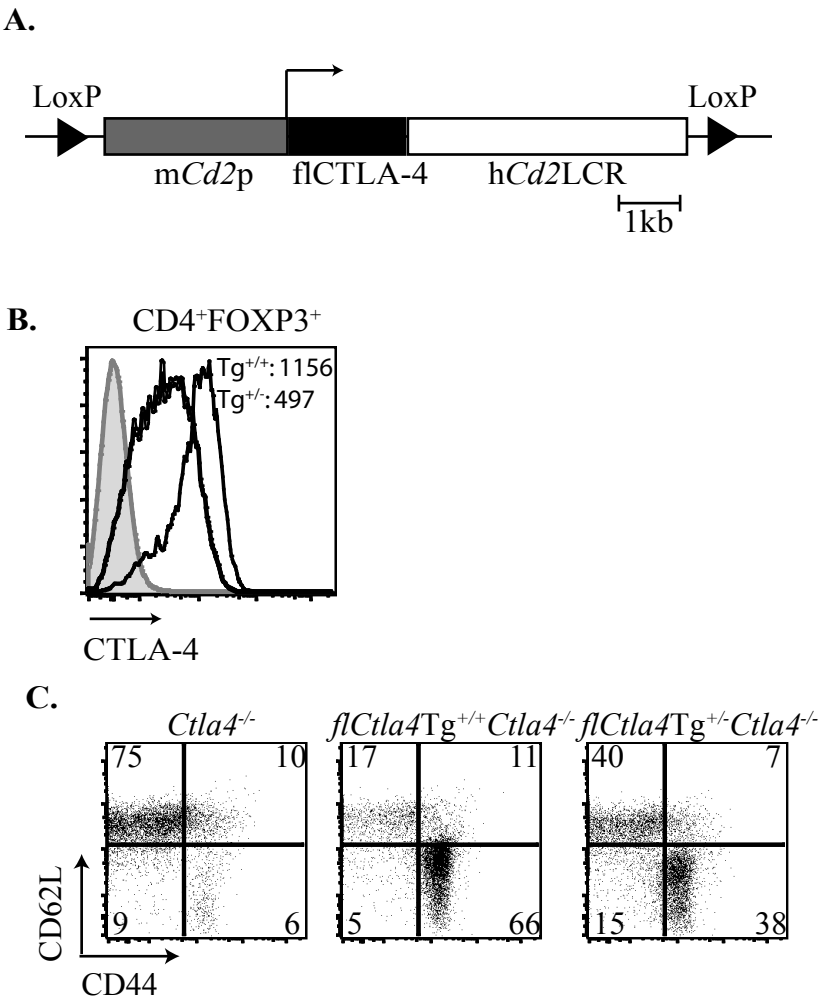

Figure II-12: Generation and characterization of *flCtla4*Tg*Ctla4*$^{-/-}$ mice

Figure II-12: Generation and characterization of *flCtla4*Tg*Ctla4*$^{-/-}$ mice.

A. Full-length *Ctla4* cDNA was cloned 3` of the *Cd2* promoter. Lox P sites were introduced at 5` and 3` ends of Tg construct to generate *flCtla4*Tg^{+} mice. **B.** Levels of expression of *flCtla4*Tg in CD4^{+}FOXP3^{+} T cells of 6-12 weeks old *flCtla4*Tg$^{+/+}$*Ctla4*$^{-/-}$ and *flCtla4*Tg$^{+/-}$*Ctla4*$^{-/-}$ mice were determined by intra-cellular staining for CTLA-4. *Ctla4*$^{-/-}$ CD4^{+} T cells were used as a negative control for CTLA-4 expression (gray histogram). Numbers in FACS plots are the mean fluorescence intensity of CTLA-4 staining normalized to *Ctla4*$^{-/-}$ control. **C.** CD4^{+}FOXP3^{-}T cells in spleens of *Ctla4*$^{-/-}$ (3-4 wks old), *flCtla4*Tg$^{+/+}$*Ctla4*$^{-/-}$ (6-12 wks old) and *flCtla4*Tg$^{+/-}$*Ctla4*$^{-/-}$ (6-12 wks old) were analyzed for expression of activation markers, CD44 and CD62L. Data are representative of 2 independent experiments with at least 2 mice in each group. While *flCtla4*Tg$^{+/+}$*Ctla4*$^{-/-}$ mice are viable, *flCtla4*Tg$^{+/-}$*Ctla4*$^{-/-}$ succumb to fatal autoimmunity by 8-10 weeks of age.

daysof age. $CD44^{lo}$ T cells that do not express CTLA-4 are most likely activated T cells that have initiated CRE expression, but have not yet up-regulated CD44 (**Fig. II-13A ;** *Bottom row,* and data not shown). Consistent with the phenotype of *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice, ablation of CTLA-4 in $FOXP3^{+}$ Treg cells resulted in conventional $CD4^{+}$ T cell activation as shown by an increased frequency of $CD44^{hi}CD62L^{lo}$ cells in secondary lymphoid organs, similar to *Ctla4*$^{-/-}$ mice (**Fig. II-13B)**. The aberrant T cell activation was detected as early as 10 days after birth (data not shown). However, unlike *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice, *Ox40*$CreTg^{+}$*flCtla4*Tg^{+}*Ctla4*$^{-/-}$ mice that sequentially lose CTLA-4 expression in Treg cells followed by the loss in activated conventional T cells succumbed to fatal autoimmunity and died by 4-6 weeks of age (**Fig. II-14A)**. Histological examination of non-lymphoid tissues revealed massive tissue infiltration in the liver, lung and heart of *Ox40*$CreTg^{+}$*flCtla4*Tg^{+}*Ctla4*$^{-/-}$ mice, indistinguishable from *Ctla4*$^{-/-}$ mice (**Fig. II-14B**), suggesting that CTLA-4 expression in aberrantly activated conventional T cells can moderate and/or alter the course of autoimmune tissue destruction.

DISCUSSION

CTLA-4 is expressed in both activated T cells and Treg cells; however, the relative contribution of CTLA-4 to the function and maintenance of these cells in controlling T cell homeostasis and activation remained undefined. In this report, we describe a mouse model of T cell subset-restricted CTLA-4 expression and demonstrate for the first time, that CTLA-4 has dual function in activated T cells and Treg cells to maintain T cell homeostasis. Expression of an *Il2*-promoter driven Tg*Ctla4* in *Ctla4*$^{-/-}$ mice results in functional CTLA-4 expression in activated T cells,

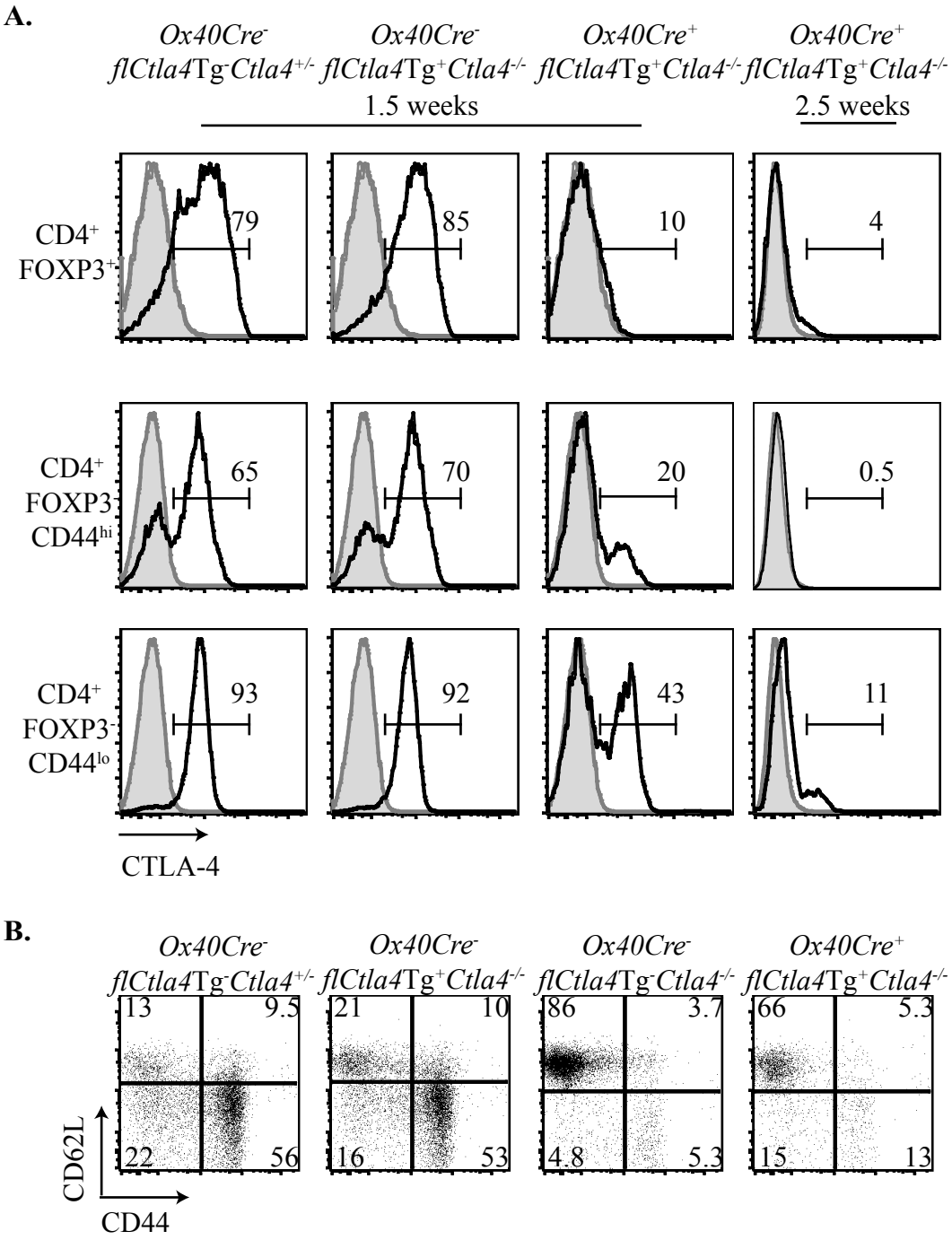

Figure II-13: Sequential loss of *flCtla4*Tg expression in Treg cells followed by conventional activated T cells in *Ox40Cre*$^{+}$*flCtla4*Tg*Ctla4*$^{-/-}$ mice

Figure II-13: Sequential loss of *flCtla4*Tg expression in Treg cells followed by conventional activated T cells in *Ox40*Cre^{+}*flCtla4*Tg^{+}*Ctla4*$^{-/-}$ mice.

A. OX40Cre mediated deletion of floxed *flCtla4*Tg substrate in T cell subsets was determined by intra-cellular staining for CTLA-4. CD4^{+} T cells in the spleens of *Ox40*Cre^{-}*flCtla4*Tg^{+}*Ctla4*$^{+/-}$ (1.5wks old), *Ox40*Cre^{-}*flCtla4*Tg^{+}*Ctla4*$^{-/-}$ (1.5wks old) and *Ox40*Cre^{+}*flCtla4*Tg^{+}*Ctla4*$^{-/-}$ (1.5wks and 2.5 wks old) were analyzed for the expression of FOXP3, CD44 and CTLA-4. (*Top row*) CD4^{+}FOXP3^{+} Treg cells, (*Middle row*) CD4^{+}FOXP3^{-}CD44^{hi} activated conventional T cells, and (*Bottom row*) CD4^{+}FOXP3^{-}CD44^{lo} conventional T cells. *Ctla4*$^{-/-}$ CD4^{+} T cells were used as a negative control for CTLA-4 expression (gray filled histogram). Data are representative of 2 independent experiments with at least 2 mice in each group. **B.** CD4^{+}FOXP3^{-}T cells in the spleen of 2.5 weeks old *Ox40*Cre^{-}*flCtla4*Tg^{+}*Ctla4*$^{+/-}$, *Ox40*Cre^{-}*flCtla4*Tg^{+}*Ctla4*$^{-/-}$, *Ox40*Cre^{-}*flCtla4*Tg^{-}*Ctla4*$^{-/-}$ and *Ox40*Cre^{+}*flCtla4*Tg^{+}*Ctla4*$^{-/-}$ were analyzed for expression of activation markers, CD44 and CD62L. Data are representative of 2 independent experiments with at least 2 mice in each group.

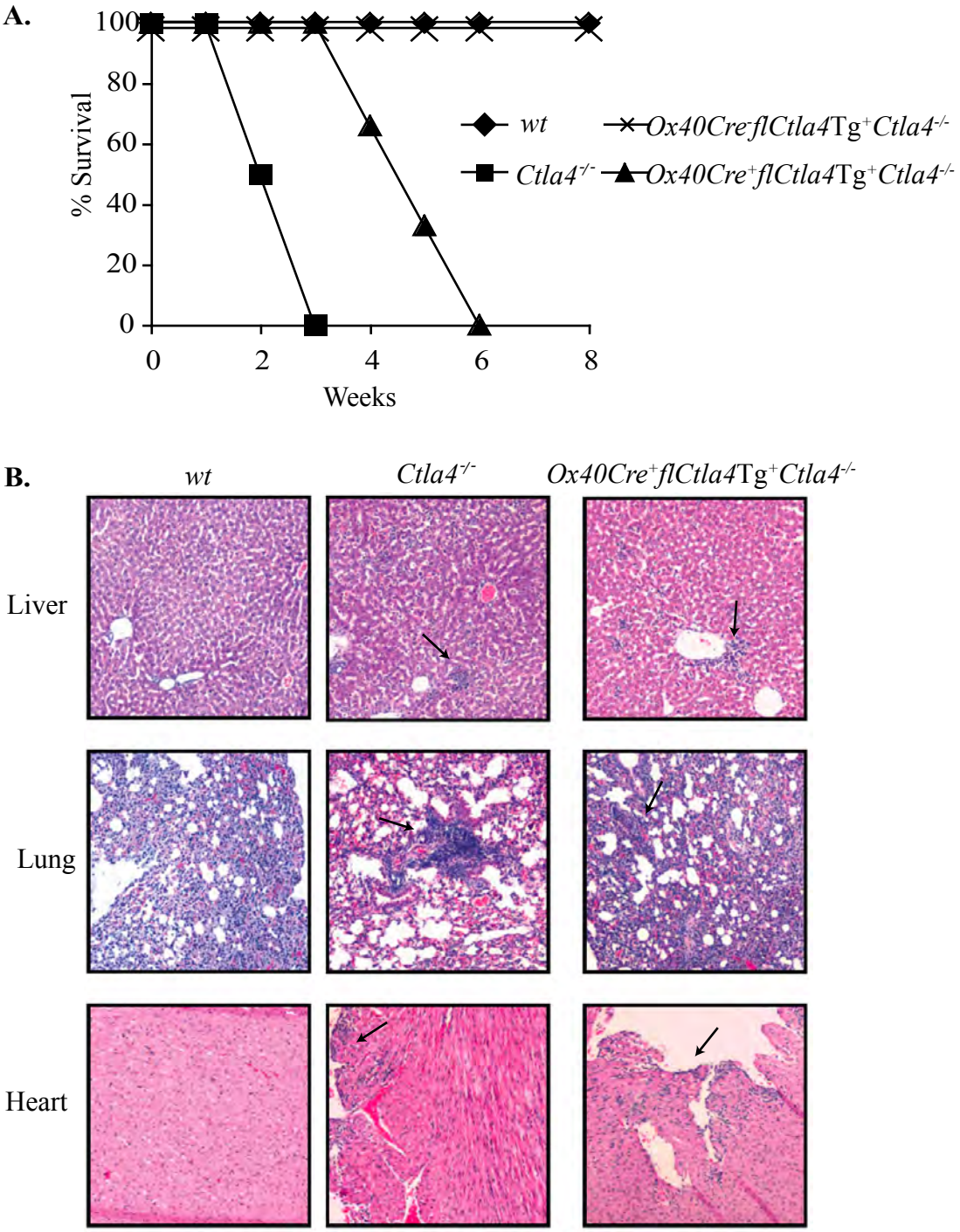

Figure II-14: Fatal autoimmunity in mice lacking CTLA-4 expression in Treg cells.

Figure II-14: Fatal autoimmunity in mice lacking CTLA-4 expression in Treg cells.

A. Mortality rate of *wt* (n=8, ♦), *Ctla4*$^{-/-}$ (n=4, ■), *Ox40*Cre^{-}*flCtla4*Tg$^{+/+}$*Ctla4*$^{-/-}$ (n=6, ✕) and *Ox40*Cre^{+}*flCtla4*Tg$^{+/+}$*Ctla4*$^{-/-}$ (n=6, ▲) mice. Results are represented as percent survival, which was calculated as 100 x (number of surviving mice/total number of mice) at each time point. **B.** Histological analysis of H&E stained sections of liver, lung and heart from 3-4 wks old *wt, Ctla4*$^{-/-}$ and *Ox40*Cre^{+}*flCtla4*Tg^{+}*Ctla4*$^{-/-}$ mice. Extensive lymphocyte infiltration was seen in sick *Ox40*Cre^{+}*flCtla4*Tg^{+}*Ctla4*$^{-/-}$ and *Ctla4*$^{-/-}$ mice. At least two sections/mouse and multiple fields were analyzed. Original magnification: 10X.

but not in FOXP3^{+} Treg cells and naïve T cells. CTLA-4-less Treg cells are functionally impaired *in vivo* and cause aberrant activation of T cells to self or environmental antigens, despite the fact that conventional T cells are capable of expressing Tg*Ctla4* within hours of activation. Conversely, provision of wt Treg cells prevented aberrant *Il2pCtla4*Tg*Ctla4*$^{-/-}$ T cell activation. Strikingly, activated lymphocytes in adult *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice that can express CTLA-4 retain CCR7 and do not home to and/or accumulate in peripheral non-lymphoid organs and cause tissue damage. In contrast, in *Ox40*CreTg^{+}*flCtla4*Tg^{+}*Ctla4*$^{-/-}$ mice, the loss of CTLA-4 on Treg cells combined with a sequential loss of CTLA-4 in aberrantly activated T cells results in fatal autoimmunity that is similar to the disease observed in *Ctla4*$^{-/-}$ mice, with ~one month delay in death. Thus, CTLA-4 on Treg cells is required to prevent inappropriate naïve T cell activation against self and/or environmental antigens and CTLA-4 expression in activated T cells is necessary to limit the pathogenic effects of inappropriately activated T cells, perhaps by preventing the infiltration of aberrantly activated T cells into tissues in *cis*, providing, literally, a "brake" in pathogenic T cell mediated destruction of self-tissues.

It was formally possible that despite the defect in CTLA-4-less Treg cells in maintaining naïve T cell homeostasis, they are partly functional and are, in fact, responsible for preventing activated T cells from infiltrating non-lymphoid organs. This possibility was conclusively ruled out by the results from mixed BMC experiments composed of *Il2pCtla4*Tg*Ctla4*$^{-/-}$: *Ctla4*$^{-/-}$ BM-derived cells, where extensive T cell infiltration into diverse tissues was evident, demonstrating that Treg cells from *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice are not able to prevent *Ctla4*$^{-/-}$ T cells from

infiltrating non-lymphoid organs. Further, although it is formally possible that some trace CTLA-4 expression in adult Treg cells of *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice is a confounding factor, we have observed that the rescue of *Ctla4*$^{-/-}$ mice from fatal autoimmunity requires a relatively high level exogenous CTLA-4Tg expression in Treg cells. Even a 50% reduction in CTLA-4 expression level in Treg cells fails to prevent T cell activation and lymphoproliferation (**Fig. II-12B, C**). Hence, functional CTLA-4 in activated T cells is responsible for preventing aberrantly activated T cells from damaging self-tissues in *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice. It is important to emphasize, however, that this conclusion does not imply that CTLA-4-less Treg cells are completely non-functional. Treg cells from *Ctla4*$^{-/-}$ mice can suppress conventional T cell activation *in vitro* (data not shown and (313)) and there are some indications, albeit not universal, that these cells can function to inhibit colitogenic T cells in a T cell transfer model (221) and that they can delay the early fatality associated with *Foxp3* null mutation (314). Conversely, mice lacking FOXP3 do succumb to rapid, fatal lymphoproliferative disease despite the fact that activated T cells can express CTLA-4 (315) suggesting that the absence of CTLA-4 on Treg cells is not functionally equivalent to the loss of Treg cells. Given the notion that Treg cells may utilize multiple effector mechanisms (316), including IL-10 (130) and TGFβ(235), to regulate T cell homeostasis and tolerance, it is not unexpected that ablation of CTLA-4 in Treg cells do not make them inert. Hence, while both *Foxp3*$^{-/-}$ and *Ctla4*$^{-/-}$ mice die of an early lymphoproliferative disease, the causes are distinct: in the former it is the loss of FOXP3-dependent functions of Treg cells, whereas in the latter it is the combined effect of the loss of CTLA-4 in Treg cells and in activated T cells. In *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice CTLA-4-less Treg cells may afford a moderation in immune

activation that allow Treg cell-independent function of CTLA-4 to take effect *in vivo*. However, other compensatory mechanisms cannot prevent naïve T cell activation in the absence of CTLA-4 on Treg cells and cannot control tissue destruction by aberrantly activated T cells.

Recently, the phenotype of conditional *Ctla4*-mutant mice (conditional KO, CKO, in BALB/c background) lacking CTLA-4 only in Treg cells (employing FOXP3-Cre Tg) was reported (222). These mice succumb to a delayed fatal lymphoproliferative disease and die by 7-10 weeks of age. This result strongly supports a major conclusion in our reports (Friedline et al. J. Exp. Med. in press and this work) derived from the analysis of several very different model systems that CTLA-4 functions centrally in Treg cells to regulate naïve T cell activation. Further, the 4-6 week survival benefit when CTLA-4 can still be expressed in Tconv cells of the CKO mice is also consistent with a role for CTLA-4 in non-Treg cells. However, an obvious question is why *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice survive so much longer. The disease course in *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice is not simply a delay in pathogenesis since even in Il2pCtla4TgCtla4$^{-/-}$ mice suffering from fulminant enteritis, many other non-lymphoid tissues are spared from T cell infiltration. Although this difference may be arising from the difference in genetic background (our mice are B6) or possible ablation of FOXP3 in other tissues of CKO mice that can cause accelerated death, we suspect that the most likely reason is the transient expression of Tg*Ctla4* in FOXP3+ thymocytes in *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice (**Fig. II-4B**). These residual, transient FOXP3+ cells may moderate the pace of aberrant T cell activation, and more importantly, dampen overwhelming inflammation associated with *Ctla4*$^{-/-}$ or *Foxp3*$^{-/-}$

mice that may override the effects exerted by CTLA-4 expression in aberrantly activated T cells. This hypothesis is currently being tested.

This report does not address the mechanism with which CTLA-4 expression on aberrantly activated T cells can regulate their potential destructive actions. However, there is one other model system in which similar dual activity of CTLA-4 has been suggested. In *Ctla4*$^{-/-}$ mice, expression of a CTLA-4 Tg lacking the cytoplasmic signaling domain of the protein resulted in aberrant T cell activation and lymphoproliferation (211). However, activated T cells did not accumulate in non-lymphoid tissues and the mice were viable. Given that one major function of CTLA-4 in conventional T cells is to inhibit CD28 signals (200), which has been shown in several situations to regulate homing of T cells (300), a straightforward interpretation is that CTLA-4 can compete with CD28 for B7 ligand binding on APCs, and that this mode of inhibition does not require biochemical signaling initiated from the CTLA-4 cytoplasmic region. We propose that this CTLA-4-cytoplamic tail-independent inhibition of CD28 signaling in aberrantly activated conventional T cells is contributing to the lack of tissue infiltration in *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice. In contrast, the function of CTLA-4 in Treg cells to maintain naïve T cell homeostasis requires CTLA-4-cytoplasmic tail-mediated signals.

Collectively, we demonstrate a dual function of CTLA-4 in regulating T cell tolerance that can account for the pathologies observed in mice lacking normal expression of CTLA-4. CTLA-4 is absolutely required on Treg cells to regulate the aberrant activation of conventional naïve T cells to self or environmental antigens in

the periphery *in trans* irrespective of whether or not conventional T cells can express CTLA-4. In addition, *in cis*, CTLA-4 expression in aberrantly activated T cells can modulate rampant autoimmune tissue destruction and alter the disease course, but this function in activated conventional T cells appears to require some moderation in the pace of aberrant T cell activation and is insufficient to completely protect all tissues in the animals as they age, since fatal enteritis eventually sets in.

Materials and Method

Mice

The IL-2P/GFP/CD2 transgene construct was a kind gift from Casey Weaver (307). Following EcoR1 and Xba1 digestion, the 1.4kb full-length CTLA-4 cDNA construct from SKCwt5/10 was inserted in place of GFP in BOB1cwt10 plasmid containing IL-2 transgene. The 9.6kB IL-2P/CTLA4/CD2 transgene was isolated from pIL-2P(hs65TGFP)-hC2 following Not1 and Sal1 digestion and injected by Umass Transgenic Facility to generate transgenic mice. Transgene-positive founders were identified by PCR analysis. 4 different founder lines were established of which 2 were chosen for crossing with *Ctla4*$^{+/-}$ mice to generate *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice. Some mice were also crossed to *5C.C7*TCRTg*Ctla4*$^{-/-}$*Rag*$^{-/-}$ mice to generate *5C.C7*TCRTg^{+}*Il2pCtla4*Tg^{+}*Ctla4*$^{-/-}$*Rag*$^{-/-}$. In addition, the 1.4kb full-length CTLA-4 cDNA construct was inserted 3` of the *Cd2* promoter (317) and Lox P sites were introduced at the 5` and 3` ends to generate floxed *Cd2pflCtla4*Tg^{+} (*flCtla4*Tg) mice (**Supplemental Fig II-12A**). *flCtla4*Tg^{+} mice on the B6 background were then crossed with *Ctla4*$^{+/-}$ mice to generate *flCtla4*Tg^{+}*Ctla4*$^{-/-}$ mice. Conditional deletion of *Ctla4*Tg in FOXP3^{+} T cells and activated T cells was achieved by crossing

*flCtla4*Tg*Ctla4*$^{-/-}$ mice with OX40-Cre mice (a kind gift from Dr. Nigel Killeen, UCSF). *Rag1*$^{-/-}$ and C57Bl/6 mice were obtained from Jackson Laboratories. All mice used in these experiments were housed in a specific pathogen-free rodent barrier facility. All experiments were approved by the University of Massachusetts Medical School Institutional Care and Use Committee.

Antibodies, Flow cytometry and cell sorting

Fluorescently labeled Abs specific to CD4, CD8a, CD44, CD62L, CD69, CTLA-4, CD103, CD5, BrdU, a4b7, and Ly5.2 were purchased from BD Pharmingen and Abs specific to FOXP3, CD25, IL-7R and GITR, were purchased from eBiosciences. Intra-nuclear FOXP3 and intracellular CTLA-4 stainings were performed according to the manufacturers' protocol (eBiosciences and BD, respectively). Surface CTLA-4 staining was performed at 37^0C for 30 minutes. All data were acquired on an EPICS XL cytometer (BD-Coulter, Hialeah, FL) or LSRII (BD), and analyzed using FlowJo software (Treestar, San Carlos, CA). Peripheral T cell subsets including $CD4^+CD25^-$ conventional T cells and $CD4^+CD25^+$ Treg cells were sorted to greater than 95% purity using MoFlo (Cytomation) cell sorter.

RT-PCR

For reverse transcription-polymerase chain reaction (RT-PCR), total RNA was isolated from purified cells (Trizol Reagent, Invitrogen) and cDNA prepared using an Omniscript RT-PCR kit (Qiagen). For semi-quantitative RT-PCR (sqRT-PCR), 3-fold serial dilutions of cDNA were prepared. PCR primers used were: *Il2*-for 5`-TCA AGC TCT ACA GCG GAA GA-3` and *il2*-rev 5`-TGA AAT TCT CAG CAT CTT

CCA A-3`; *TgCtla4*-for 5`-ATG GCT TGT CTT GGA CTC CGG AG-3` and Tg*Ctla4*-rev 5`-TCA GTT GAT GGG AAT AAA ATA AGG CT-3`; *Actb*-for 5`-CTA GGC ACC AGG GTG TGA TGG-3` and *Actb*-rev 5`-TCT CTT TGA CAC GCA CGA-3`.

In vitro T cell activation and proliferation

Sorted $CD4^+CD25^-$ T cells were activated with Sepharose beads coated with anti-CD3 (500A2; eBiosciences) (0.5μg/ml), anti-CD28 (37N; eBiosciences) (1.0μg/ml) and either anti-hamster isotype control Ab or anti-CTLA4 (UC10.4F1; BD Biosciences) (5.0μg/ml) Ab in complete DMEM (10% FBS, 50μM 2-ME, 2mM L-glutamine, 20mM Hepes, 0.1mM Non-Essential Amino Acids) for 72h. Beads were coated with Ab at 37^0C for 1h in sterile PBS.

BrdU labeling and detection

Mice received twice daily injections of 1mg of BrdU i.p. for two days before sacrifice. Cells were stained for surface markers as usual, fixed and then stained with anti-BrdU-FITC Ab (eBiosciences) as described in the accompanying article by Friedline et al.

Colitis

5 x 10^5 $CD4^+CD25^-$ naïve T cells from *wt* mice were transferred to lymphocyte-deficient (*Rag1*$^{-/-}$) mice to induce colitis. To test the function of Treg cells in preventing colitis, 2 x 10^5 $CD4^+CD25^+$ Treg cells from *wt,* *Ctla4*$^{-/-}$ and *Il2pCtla4*Tg*Ctla4*$^{-/-}$ mice were co-injected. Mice were weighed weekly and examined

for signs of colitis and wasting. Mice were considered unhealthy if they displayed any of these signs: eye inflammation, scruffy coat, weight loss and loose stools. Mice were sacrificed and colons removed at 4-5 weeks after T cell reconstitution for histological examination.

Mixed bone marrow chimeras (BMC)

BM was flushed from the femurs and tibias of donor mice and depleted of $CD4^+$ and $CD8^+$ T cells using magnetic Dynal beads. 4 x 10^6 BM cells were injected into lightly irradiated (300 rads) *Rag1*$^{-/-}$ mice via the tail vein. Mice were bled periodically to determine reconstitution of the peripheral T cell pool. Mice were sacrificed and analyzed at >12 weeks post transfer.

Histological examination

Organs were removed and fixed in 10% formalin. Four microns paraffin embedded sections were cut and stained with hematoxylin and eosin (H&E). At least 4 sections and multiple fields were analyzed.

Intestinal intra-epithelial lymphocyte (iIEL) preparation

Intestinal IELs were prepared using discontinuous density gradient centrifugation. Briefly, small intestines were slit longitudinally and cut into 1 cm pieces after removing Peyer's Patches and faecal matter. Intestines were incubated in 2% RPMI at 37^0C with gentle stirring for 30 minutes. Tissues were transferred into 50ml conical tubes and shaken vigorously for 15 seconds. The cells were filtered over a steel mesh and then re-suspended in 3ml of 40% Percoll. The suspension was layered onto 70%

Percoll and centrifuged at 300g for 30 minutes at 20^0C. The interface, which contains IELs, was removed and washed in complete RPMI. iIEL preparations routinely contained 75-80% viable cells.

Statistical Analysis

All data are representative of at least 3 independent experiments. P values were calculated using Student's t-test.

CHAPTER III

Dab2 programs potential TGFβ responsiveness of T cells during early development and is required for Foxp3^{+} regulatory T cell function.

Attributions and Copyright information

The data presented in this Chapter were collected in collaboration with Dr. Hai Nguyen, Nidhi Malhotra and Dr. Randall Friedline. Specific contributions are as follows:

Dr. Hai Nguyen: Figure III-1A, C, E; Figure III-3A, Figure III-9E

Nidhi Malhotra: Figure III-2B, Figure III-3B

Dr. Randall Friedline: Figure III-4A

The *Dab2*$^{fl/fl}$ mice were a kind gift from Dr. Jonathan Cooper (318).

Much of the data in this Chapter is part of a manuscript that has been submitted for publication.

Introduction

The importance of a reservoir of T cells capable of inhibiting the activities of potentially self-destructive effector T cells is now widely appreciated. The best studied among several different types of suppressor T cell subsets is the thymus-derived regulatory $CD4^+$T cell subset, called natural regulatory T (Treg) cells. These cells are distinguished from conventional T cells primarily based on their expression of CD25 (IL-2Rα) and the transcription factor FOXP3 (88), which can be induced in developing Treg cells by TGFβ. In humans and mice, mutations in the *Foxp3* gene lead to fatal, early onset autoimmunity, mirroring the breakdown in T cell tolerance in TGFβ-deficient animals (87, 238).

$FOXP3^+$ Treg cells can also be generated from naïve T cells in the presence of TGFβ *in vitro* (97) or with suboptimal TCR stimulation *in vivo* (319). TGFB-dependent *Foxp3* induction in T cells requires SMAD3, which acts in concert with other modulators, most notably NFAT that is activated by TCR signaling, to initiate and maintain *Foxp3* transcription (115). SMAD recruitment and phosphorylation by the TGFβ receptor complex is controlled at multiple levels and there are a myriad of adaptors and inhibitors that fine-tune the kinetics, duration and nuclear residency of activated SMADs (320). Moreover, the quantity of TGFβ signals is a critical parameter controlling naïve $CD4^+$ T cell differentiation, with high concentrations of TGFβ required to commit to the $FOXP3^+$ Treg lineage and low concentrations of TGFβ leading to the generation of an alternate T cell subset producing pro-inflammatory cytokines such as IL-17 termed Th17 cells (273).

Several mechanisms for Treg cell-mediated suppression of effector T cell activation have been proposed, including the production of regulatory cytokines TGFβ. (235), IL-10 (130) and IL-35 (135), and modifications of antigen presenting cells (APCs) such that they are rendered ineffective in activating T cells (321). An emerging theme in the mechanism of Treg-mediated suppression *in vivo* is that it is highly context-dependent and likely involves fail-safe, multiple effector components. This lack of dependence on a single, dominant means of immune suppression reflects a wide spectrum of conditions in which T cell homeostasis and quiescence must be maintained and reestablished subsequent to infection.

Treg cell-restricted expression of *Foxp3* among lymphocytes and the absolute requirement for FOXP3 in Treg function have stimulated intensive investigations to determine FOXP3 target genes and proteins that are essential for Treg cell function. So far only a limited number of possible candidates have emerged: Phosphodiesterase 3b (*Pde3b*) inactivates cAMP and its expression is inhibited by FOXP3 (101). Enforced over-expression of *Pde3b* in Treg cells leads to defects in their maintenance *in vivo*. Although the suppressive activities of $Pde3b^{+}$ Treg cells have not been described, cAMPs have been suggested to be important effector molecules of Treg cells (144). IL-35 is a newly defined cytokine expressed selectively in Treg cells. It is composed of IL-12α and IL-27β, which is encoded by *Ebi3*, a FOXP3 target gene. $Ebi3^{-/-}$ and $Il12a^{-/-}$ mice do not develop spontaneous autoimmunity, but IL-35-deficient Treg cells display impaired regulatory function *in vitro* and they are ineffective in curing established colitis in cell transfer assays *in vivo* (135).

Here, we show that *Dab2* is a target gene of FOXP3 that is critical for Treg cell function *in vitro*. Unlike previously identified targets of FOXP3 that are expressed in other peripheral lymphocyte subsets, *Dab2* expression is restricted to $CD4^+$ Treg cells. In non-lymphoid cells, *Dab2* expression is regulated by the Vitamin A metabolite all-*trans* Retinoic Acid (ATRA) (322) and it has been implicated in several critical functions in cell development and transformation, by enhancing SMAD activation during TGFβ signaling (248, 323, 324), in GAP junction functions (325), and in clathrin coated pit-mediated endocytosis of cell surface receptors (326). Homozygous mutation of *Dab2* results in early embryonic lethality (318), most likely as a consequence of defective signaling in the visceral endoderm by Nodal, a member of the TGFβ superfamily of cytokines. Analyses of mice with a T cell-restricted *Dab2*-deficiency show that they generate normal numbers of Treg cells, but these Treg cells are defective in function *in vitro* and *in vivo*. *Dab2* is also expressed during thymic T cell development in the precursors in a *Foxp3*-independent manner. We provide evidence that the disruption of DAB2 function in the precursors, but not in later thymic developmental phases, including Treg cells, alters the TGFβ signaling properties of mature conventional T cells, indicating that TGFβ responsiveness in T cells is an inherited, programmable trait regulated by *Dab2* at the precursor cell stage.

Results

Dab2 expression is restricted to $Foxp3^+$ Treg cells and early thymic precursor cells.

To identify genes regulated by FOXP3, we infected an immature αβ T cell lineage thymoma cell line ($CD4^+CD8^+$, NFC) with a retrovirus expression vector

containing *Foxp3* cDNA. We then performed global gene expression profiling to determine genes whose expression was changed by the ectopic *Foxp3* expression (data not shown). One gene among only a handful that was altered in expression in $FOXP3^+$ NFC cells was *Dab2* (Drosophila Disabled homolog-2, also called Doc2, differentially expressed in ovarian carcinoma 2), as illustrated by a confirmatory RT-PCR assay (**Fig. III-1A**). Outside the hematopoietic compartment, *Dab2* is widely expressed, especially in developing vasculature, placenta, adrenal gland, kidney and smooth muscle (327). Among hematopoietic cell subsets, *Dab2* is expressed in platelets (328), murine macrophages (329), human lymphoid PB-$BDCA4^+$ DCs (Novartis, http://wombat.gnf.org) and in the thymus (246). We found that thymic precursor cell subset DN3 ($CD4^-CD8^-CD44^-CD25^+$ **Fig. III-1B**) is the predominant population expressing *Dab2* in the thymus, followed by DN4 ($CD4^-CD8^-CD44^-CD25^-$) cells that express 10-30% of the amount detected in DN3 cells. *Dab2* mRNA is detected at low levels in DP and is absent in CD8SP cells (**Fig. III-1B** and data not shown). Analysis of CD4SP ($CD4^+CD8^-$) cells sorted based on FOXP3 expression from the FOXP3-IRES-GFP reporter mice (285) showed that *Dab2* expression in CD4SP thymocyte is restricted to $FOXP3^+$ cells (**Fig. III-1B**).

In peripheral lymphocyte subsets, RT-PCR assays showed that *Dab2* is exclusively expressed in the $CD4^+CD25^+$ Treg cells that are $FOXP3^+$ (**Fig. III-1C** and data not shown). *Dab2* is also expressed in TGFβ-induced $FOXP3^+$ Treg cells *in vitro* and its expression is enhanced by RA, an inducer of Dab2 expression in non-lymphoid cells (**Fig. III-1D**). In T cells, RA has been shown to synergize with TGFβ to promote Treg cell generation at the expense of inflammatory Th17 subset

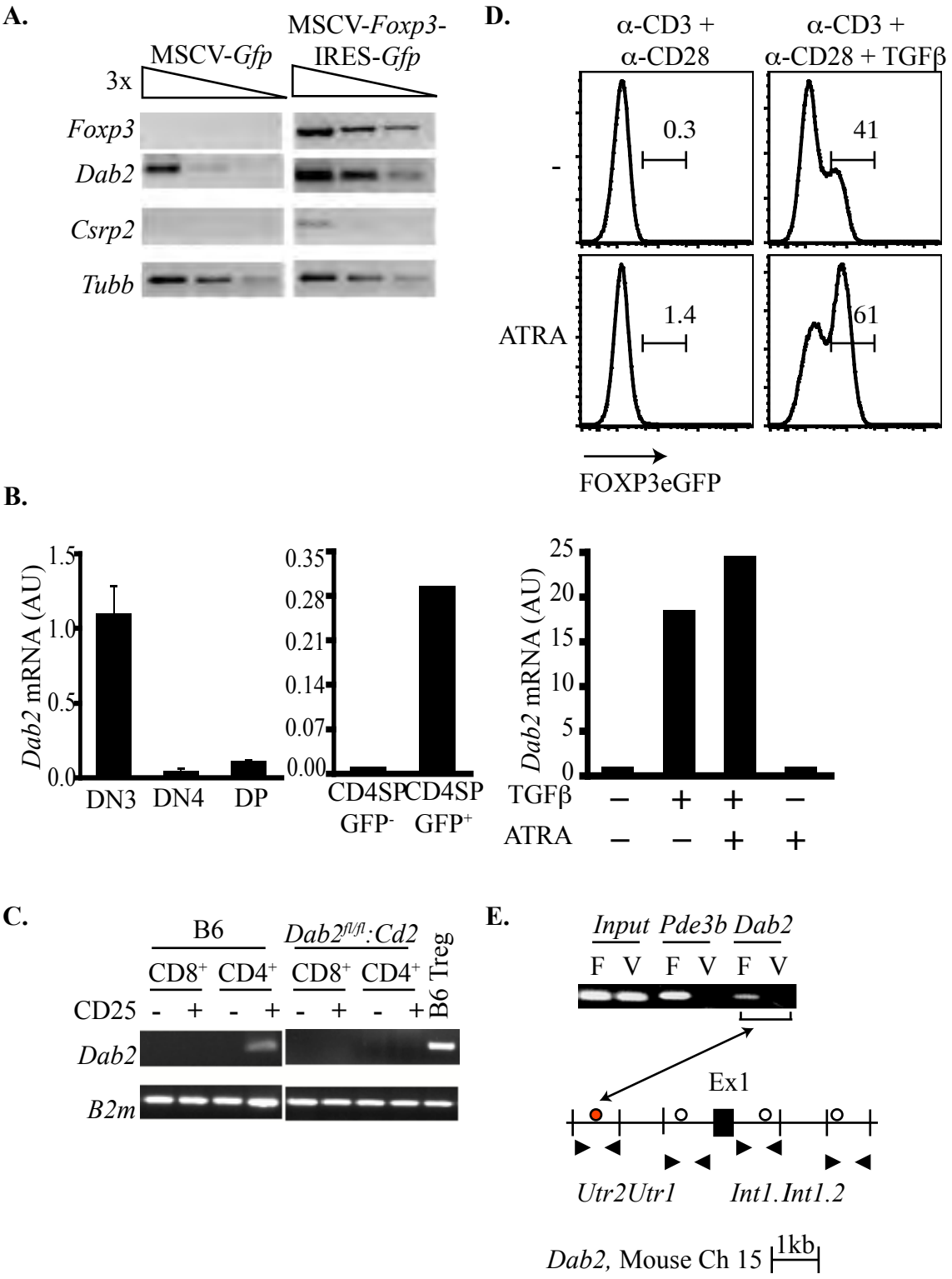

Figure III-1: FOXP3 dependent and independent *Dab2* expression in regulatory T cells and thymic precursor cells

Figure III-1: Foxp3 dependent and independent *Dab2* expression in regulatory T cells and thymic precursor cells

A. Semi-quantitative RT-PCR (sqRT-PCR) analysis of *Dab2* expression (four-fold serial dilution of cDNA) in NFC cells that were infected with a control retroviral vector (MSCV-GFP) and a retroviral vector carrying the full-length *Foxp3* cDNA (MSCV-*Foxp3*-IRES-GFP). *Csrp2*, Cysteine-rich protein 2, is a potential TGFβ-regulated gene that has also been classified as a Treg cell signature gene. It is marginally induced in expression in conventional peripheral $CD4^+$ T cells transduced with a *Foxp3* expression vector. **B.** Real-time PCR analysis of *Dab2* expression in thymic subsets: **DN3**, $CD4^-CD8^-CD44^-CD25^+$; **DN4**, $CD4^-CD8^-CD44^-CD25^-$; **DP**, $CD4^+CD8^+$; **CD4SPGFP$^-$**, $CD4^+CD8^-Foxp3^-$; **CD4SPGFP$^+$**, $CD4^+CD8^-Foxp3^+$. All data are normalized to *Actb* mRNA expression and are representative of 3 individual experiments. ***AU***: Arbitrary Units **C.** RT-PCR analysis of *Dab2* expression (one of five independent experiments) in peripheral $CD4^+$ and $CD8^+$ lymphocyte subsets, sorted by CD25 expression, from B6 and *Dab2$^{fl/fl}$:Cd2* mice. B6 Treg: sorted $CD4^+CD25^+$ T cells from B6 mice. **D.** FACS histograms showing *Foxp3-GFP* reporter expression in sorted $CD4^+GFP^-$ naïve T cells stimulated with anti-CD3 and anti-CD28 in the presence or absence of TGFβ (2ng/ml) and all-trans retinoic acid (ATRA, 100nM); Real-time PCR analysis of *Dab2* mRNA expression, normalized to *Actb* mRNA, in each stimulation condition. Data are representative of 3 individual experiments. ***AU***: Arbitrary Units **E.** Chromatin-immunoprecipitation assay to determine FOXP3 binding to *Dab2* gene regulatory element; four FOXP3 consensus binding sites were identified (circles), two upstream of exon 1 (*Utr1*, *Utr2*) and two in intron 1 (*Int1.1*, *Int1.2*). ChIP assay (one of two independent experiments) on

PMA/Ionomycin activated NFC-*Foxp3* (F) and NFC-vector alone (V) cells using anti-FOXP3 Ab showed FOXP3 binding only to *Utr2* region (filled circle). The other three sites were not occupied by FOXP3. Anti-FOXP3 antibody binding to *Pde3b* locus was used as a positive control.

production (296). These results collectively show that FOXP3 regulates *Dab2* expression in T cells. However, the expression of *Dab2* in TN cell subsets that do not express *Foxp3* (88) indicates that there may be other regulators of *Dab2* expression in the thymus (330).

To determine whether FOXP3 directly regulates *Dab2* transcription, chromatin immunoprecipitation (ChIP) experiments were performed. Relevant FOXP3 consensus binding sites in the regulatory sequences of *Dab2* were selected based on conservation between the mouse and human *Dab2* genomic loci. Among four such conserved consensus binding sites tested only one located in the 5' untranslated region, ~2.3 kb upstream of the transcriptional site of the *Dab2* gene, was found to be associated with FOXP3 in $FOXP3^+$ NFC cells (**Fig. III-1E**). These results identify *Dab2* as a potential direct target gene of FOXP3.

Dab2 deficiency results in altered Treg cell function

The expression pattern of *Dab2* in T cells combined with the known function of DAB2 as a positive regulator TGFβ signaling in non-lymphoid cells strongly suggested that DAB2 regulates TGFβ responsiveness of T cells and contributes to Treg cell function downstream of FOXP3. To determine the function of DAB2 in thymic precursor cells and in Treg cells, we generated mice lacking *Dab2* specifically in T cells in a developmental-stage controlled manner. We analyzed both *Cd2* promoter-Cre Tg^+ x $Dab2^{fl/fl}$ (referred to as $Dab2^{fl/fl}$*:Cd2*) and *Cd4* promoter-Cre Tg^+ x $Dab2^{fl/fl}$ ($Dab2^{fl/fl}$*:Cd4*) mice that delete the floxed *Dab2* gene during early ($CD3^-CD4^-CD8^-$ TN thymocytes) and late ($CD4^+CD8^+$DP, αβ T cell lineage

thymocytes) stages of intrathymic T cell development, respectively. In both conditional *Dab2* knock-out (CKO) lines, Treg cells completely lacked *Dab2* expression (**Fig. III-1C** and data not shown).

We first assessed *Dab2* function in Treg cells. Mice lacking DAB2 in Treg cells were healthy in appearance and did not suffer from overt autoimmunity even at 10-12 months of age, suggesting that the mutant Treg cells were capable of maintaining peripheral T cell tolerance in unmanipulated mice. The development of Treg cells was relatively normal in *Dab2$^{fl/fl}$:Cd4* and in *Dab2$^{fl/fl}$:Cd2* mice. There was a 2-fold increase in the frequency of CD4^{+}Foxp3^{+}T cells in the thymus (**Fig. III-2A**) and spleen of *Dab2$^{fl/fl}$:Cd2*, but not *Dab2$^{fl/fl}$:Cd4* mice (**Fig III-2B**). This relative enhancement of FOXP3^{+} cells in the CD4^{+} T cell subset in *Dab2$^{fl/fl}$:Cd2* mice increased as mice aged, with nearly half of the CD4^{+} T cells expressing FOXP3 by 9 months of age (data not shown). However, at all ages the total numbers of FOXP3^{+} T cells were comparable to *wt* mice (data not shown), indicating a loss of other lymphocyte subsets rather than an increase in the production of Treg cells *per se* in *Dab2$^{fl/fl}$:Cd2* mice (see below). Phenotypically, Treg cells from both *Dab2$^{fl/fl}$:Cd2* and *Dab2$^{fl/fl}$:Cd4* mice appeared more activated, as indicated by a higher frequency of CD44 expression, compared to *wt* Treg cells (**Fig. III-2C**). DAB2 has been shown to bind to the clathrin-coated pit adaptor protein AP2, which regulates CTLA-4 expression by endocytosis (326). However, we did not detect any changes in total or surface CTLA-4 expression in these Treg cells (**Fig. III-2C**). Further, other distinguishing cell surface markers of Treg cells, such as GITR and IL-7R (data not shown), were normal in expression on *Dab2* CKO Treg cells, but a greater frequency

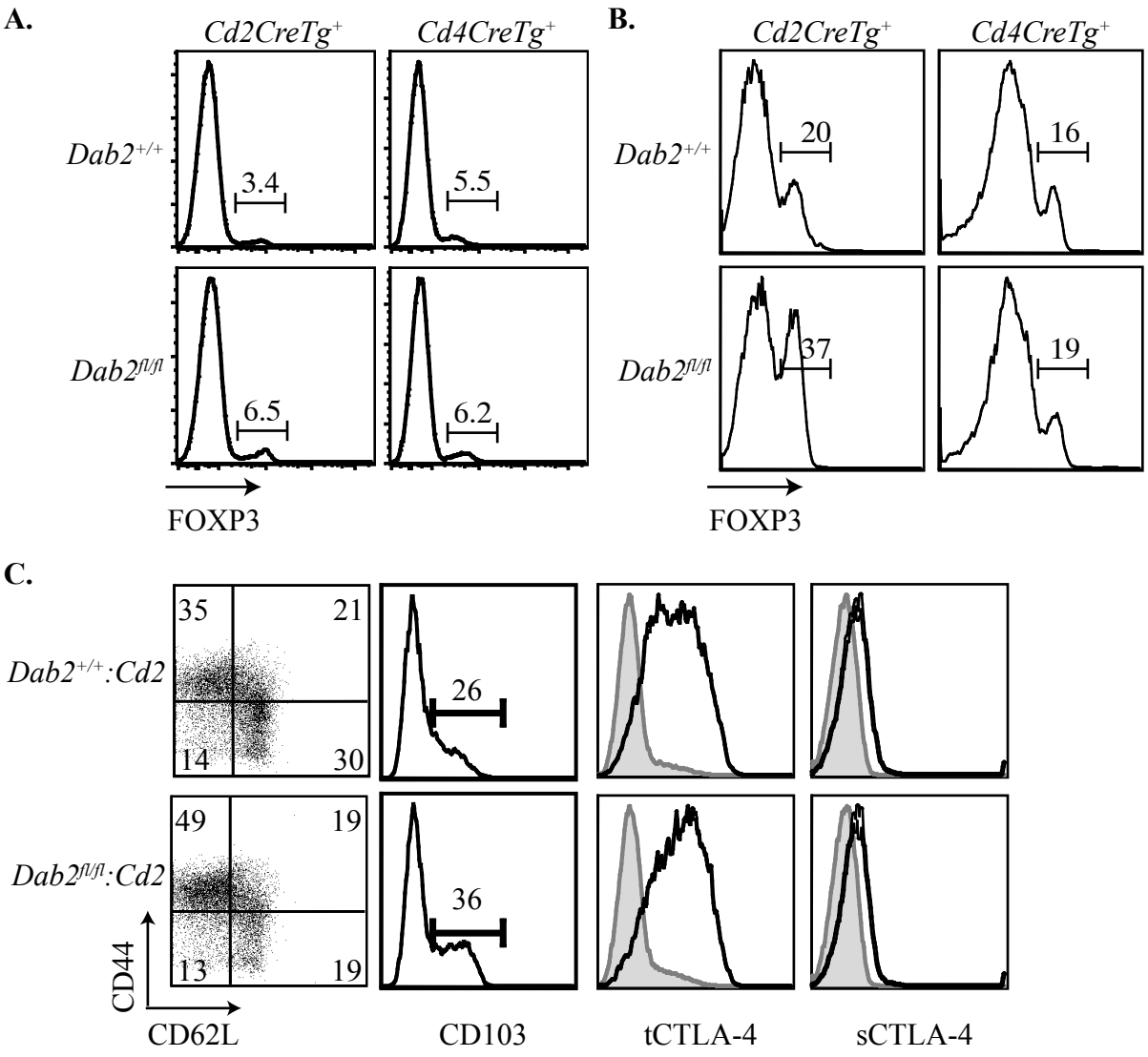

Figure III-2 Development and phenotype of Treg cells in *Dab2$^{fl/fl}$:Cd2* and *Dab2$^{fl/fl}$:Cd4* mice

Figure III-2 Treg cell development and phenotype in *Dab2$^{fl/fl}$:Cd2* and *Dab2$^{fl/fl}$:Cd4* mice.

A. A representative flow cytometric analysis of thymocytes from littermate *wt*, *Dab2$^{fl/fl}$:Cd2* and *Dab2$^{fl/fl}$:Cd4* mice for intracellular expression of FOXP3 in CD4^{+} T cells. **B.** A representative flow cytometric analysis of splenocytes from littermate *wt*, *Dab2$^{fl/fl}$:Cd2* and *Dab2$^{fl/fl}$:Cd4* mice for intracellular expression of FOXP3 in CD4^{+} T cells. **C.** Flow cytometric analysis of expression of CD44, CD62L, CD103, total CTLA-4 and surface CTLA4, on CD4^{+}FOXP3^{+}cells from *wt* and *Dab2$^{fl/fl}$:Cd2* splenocytes. Shaded histograms represent CD4^{+}FOXP3^{-} cells

of them expressed CD103 (**Fig. III-2C**), a TGFβ and ATRA regulated integrin molecule (297) and a marker of effector Treg cells that controls the homing of Treg cells to the gut mucosa (331).

Given that most proposed mechanisms of Treg function are not exclusively required to enforce T cell self tolerance in un-manipulated mice (316), the lack of overt disease in *Dab2* CKO mice was not necessarily indicative of a lack of role for DAB2 in Treg cell function. Thus, we further assessed the functional competence of *Dab2*-deficient Treg cells using the standard assays. We first tested the ability of purified $CD4^+CD25^+$ Treg cells from $Dab2^{fl/fl}$*:Cd2* and $Dab2^{fl/fl}$*:Cd4* mice to suppress naïve T cell proliferation *in vitro*. Whereas *wt* Treg cells suppressed the proliferation of responder T cells in a Treg cell number-dependent manner, Treg cells from $Dab2^{fl/fl}$*:Cd2* mice were completely unable to suppress effector T cell proliferation. (**Fig III-3A**). A similar defect was observed with Treg cells from $Dab2^{fl/fl}$*:Cd4* mice (data not shown).

To determine the mechanistic parameters that contribute to the defective function of *Dab2*-deficient Treg cells, we examined some of the potential candidates of Treg cell-mediated immune suppression *in vitro*. Treg cells from $Dab2^{fl/fl}$*:Cd2* and $Dab2^{fl/fl}$*:Cd4* mice produced normal amounts of IL-10 as measured by ELISA (**Fig. III-3B**) and RT-PCR, expressed comparable amounts of *TGFβ* and *Ebi3* at the transcriptional level and expressed normal amounts of Granzyme B protein (data not shown). There were also no dramatic alterations in the expression of select TGFβ-regulated genes (Data not shown). Recently, it was shown that Treg cells suppress target cell

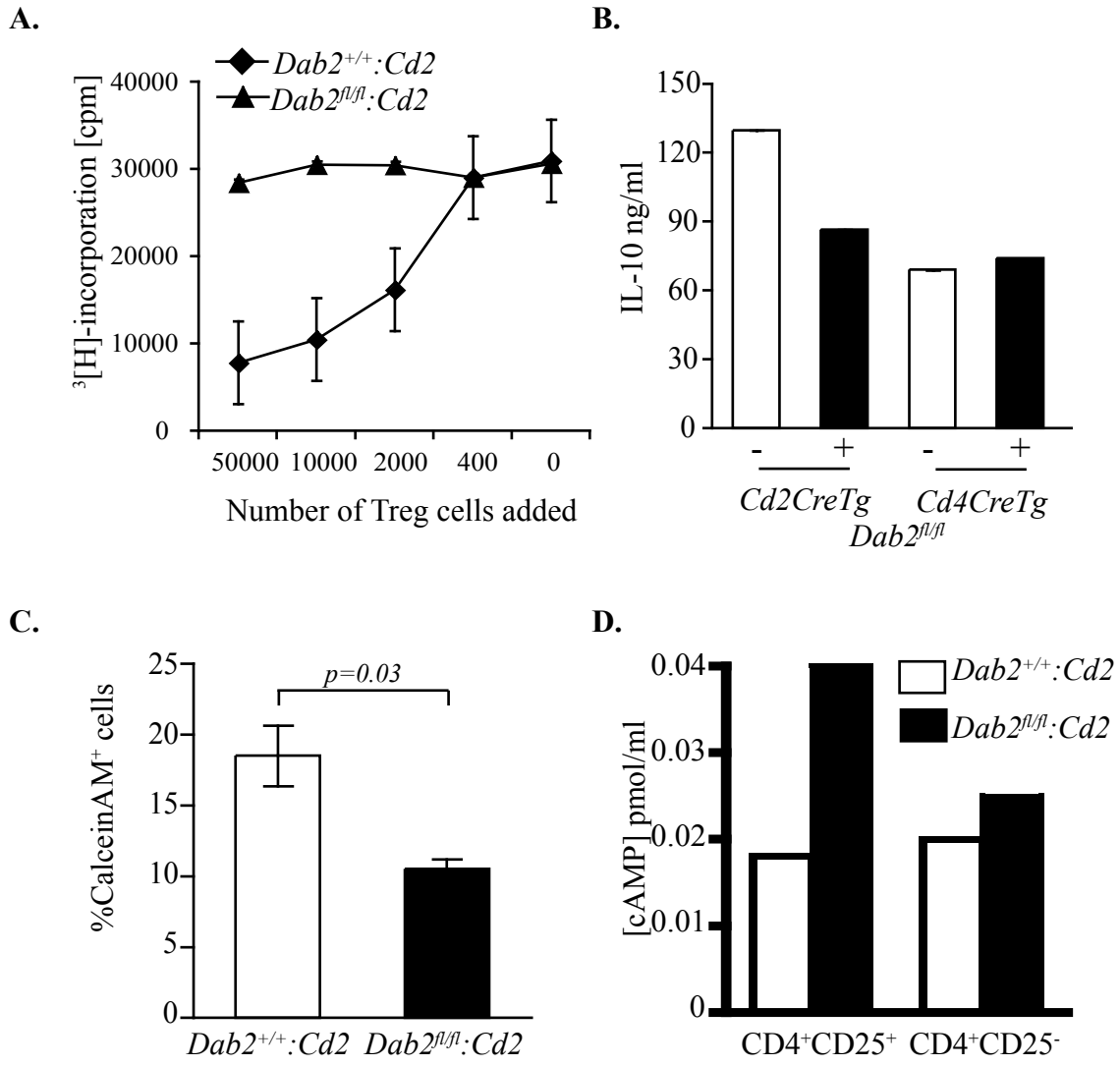

Figure III-3: Treg cells from *Dab2*-deficient mice are not functional *in vitro*

Figure III-3: Treg cells from *Dab2*-deficient mice are not functional *in vitro*

A. Proliferation of responder $CD4^+$ effector T cells co-cultured with varying numbers of sorted and pre-activated $CD4^+CD25^+$ cells from *wt* or *Dab2$^{fl/fl}$:Cd2* mice. Cells were stimulated with plate bound anti-CD3 plus anti-CD28 and proliferation was measured by ^3H thymidine incorporation at 65 hrs post-activation. One of six independent experiments with similar results is shown. **B.** IL-10 production by anti-CD3 plus anti-CD28 stimulated $CD4^+CD25^+$ cells from *wt, Dab2$^{fl/fl}$:Cd2* and *Dab2$^{fl/fl}$:Cd4* mice as measured by standard ELISA. One of two independent experiments with similar results is shown. **C.** $CD4^+$T cells from Ly5.1 congenic mice were co-cultured with Calcein AM-loaded *wt* or *Dab2$^{fl/fl}$:Cd2* $CD4^+CD25^+$ Treg cells and stimulated with plate-bound anti-CD3 plus anti-CD28. Gap-junction function was assessed by determining the frequency of $Calcein^+Ly5.1^+$ cells at 16 hours of culture. Data are averaged over 3 independent experiments. **D.** Cytosolic cAMP concentrations in sorted $CD4^+CD25^+$ Treg and $CD4^+$ $CD25^-$ conventional T cells from *wt* and *Dab2$^{fl/fl}$:Cd2* mice as measured by a standard cAMP ELISA. One of two independent experiments with similar results is shown.

proliferation and activation by transferring cAMP through GAP junctions to effector T cells (144). Since DAB2 is known to interact with connexins that make up the GAP junctions (325), we tested whether *Dab2*-deficient Treg cells were poor suppressors *in vitro* because they had deficiencies in GAP junction-mediated intercellular communication (GJIC). We labeled Treg cells from *wt* and and *Dab2*$^{fl/fl}$*:Cd2* mice with a GAP junction transferable dye, Calcein AM, and co-cultured these with congenically marked *wt* responder cells. *Dab2*-deficient Treg cells were unable to transfer the dye as efficiently as *wt* Treg cells (**Fig. III-3C**). In addition *Dab2*-deficient Treg cells expressed higher amounts of intracellular cAMP compared to *wt* Treg cells (**Fig. III-3D**). These results suggest that one impaired function in *Dab2*-deficient Treg cells involves the GJIC, while other mechanisms of Treg cell-mediated suppression examined appeared largely intact.

Next, we determined the ability of purified Treg cells from *Dab2*$^{fl/fl}$*:Cd2* and *Dab2*$^{fl/fl}$*:Cd4* mice to control co-injected pathogenic naïve T cells when transferred into lymphopenic animals. In contrast to the complete lack of function of *Dab2*-deficient Treg cells *in vitro*, they were as effective as *wt* Treg cells in preventing the induction of colitis by naïve T cells transferred to *Rag1*$^{-/-}$ mice (**Fig. III-4A**). However, in a more stringent model of Treg function, *Dab2*-deficient Treg cells were incapable of moderating already established colitis (332) (**Fig. III-4B**). While *wt* Tregs efficiently reversed colitis, both *Dab2*$^{fl/fl}$*:Cd2* and *Dab2*$^{fl/fl}$*:Cd4* Treg cells were incapable of regulating lymphocyte infiltration and accumulation in the colon (**Fig. III-4C**). However, while mice receiving *Dab2*-deficient Treg cells could not maintain health as compared to mice that received *wt* Treg cells, histological analysis of colons

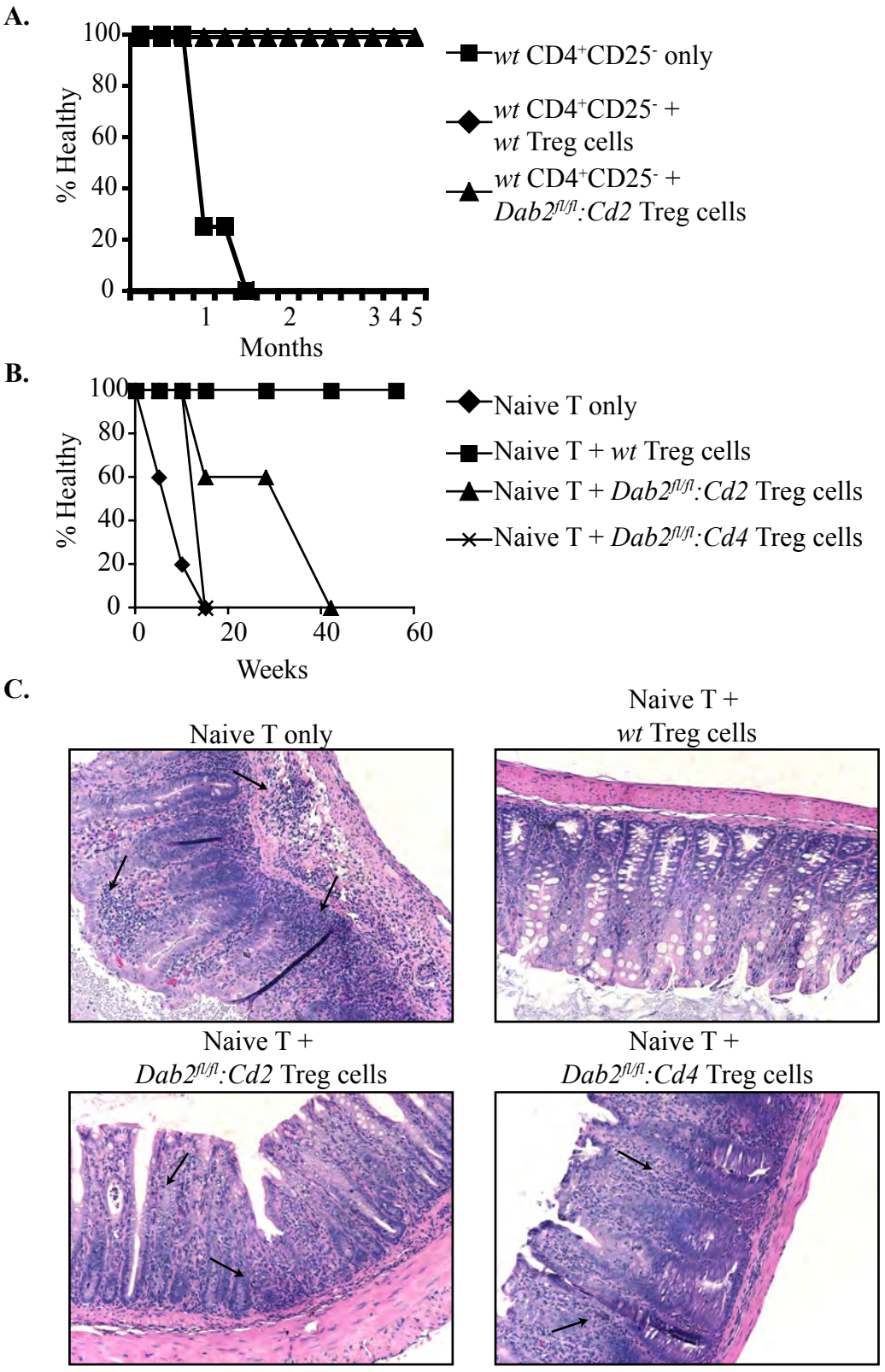

Figure III-4: *Dab2*-deficient Treg cells are functionally impaired *in vivo*

Figure III-4: *Dab2*-deficient Treg cells are functionally impaired *in vivo*

A. 4 x 10^5 sorted $CD4^+CD25^-$ T cells with or without 2 x 10^5 sorted $CD4^+CD25^+$ cells from *wt* or *Dab2$^{fl/fl}$:Cd2* mice were *i.v.* injected into *Rag1$^{-/-}$* recipients (n=4 for each group; one of three independent experiments with similar results is shown). Mice were weighed and health was monitored weekly for visible signs of colitis, inflammation or wasting. Mice were euthanized if they lost more than 25% of starting weight. **B.** 5 x 10^5 sorted naïve $CD4^+CD25^-$ $CD45RB^{hi}$ T cells were injected intra-peritoneally (i.p.) into *Rag1$^{-/-}$* recipients. Four weeks after transfer, 1 x 10^6 sorted $CD4^+CD25^+$ Treg cells from *wt, Dab2$^{fl/fl}$:Cd2* and *Dab2$^{fl/fl}$:Cd4* mice were i.p. injected and mice were weighed and health was monitored weekly for visible signs of colitis, inflammation or wasting. **C.** At 5 weeks after transfer of Treg cells, mice were euthanized and colons were prepared for histological analysis. Representative H&E stained sections of colons from mice that received naïve T cells only, naïve T plus wt Treg cells, naïve T plus *Dab2$^{fl/fl}$:Cd2* Treg cells and naïve T plus *Dab2$^{fl/fl}$:Cd4* Treg cells are shown. Original magnification is 10x.

at 2 months after Treg cell transfer showed that Dab2-deficient Treg cells could partially control some aspects of colitis like mucin-cell depletion (data not shown).

To determine whether T cell response against pathogen was perturbed in *Dab2$^{fl/fl}$:Cd2* mice, they were challenged with LCMV (clone 13, whose clearance in mice is inhibited by IL-10 (333) or acutely infected with *Leishmania major* (334). The mutant mice resolved the infections relatively normally despite a dramatic difference in the number of T lymphocytes in *Dab2$^{fl/fl}$:Cd2* mice (data not shown) and in the case of LCMV infection, a delayed CD8 T cell activation and an impaired CD4 T cell mobilization (data not shown). Whether Treg cells are responsible for the altered immune response in the LCMV model will require further studies. Collectively, these results indicate that while DAB2 is essential for Treg cell-mediated suppression of effector T cell proliferation *in vitro*, and may contribute to Treg cell-mediated immune modulation during infection, it is not absolutely required for Treg cell-mediated maintenance of T cell homeostasis and T cell tolerance *in vivo*.

Altered lymphocyte development in *Dab2$^{fl/fl}$:Cd2* mice.

While *Dab2* expression is restricted to Treg cells in the periphery, there is FOXP3-independent *Dab2* expression in early T cell precursors in the thymus (**Fig. III-1B**). To determine the function of DAB2 in precursors, we reasoned that T cell defects seen in *Dab2$^{fl/fl}$:Cd2* mice but not in *Dab2$^{fl/fl}$:Cd4* mice would identify developmental stage-specific DAB2 function (**Fig. III-5A**). This comparison was made relatively straightforward because the only lymphocyte defect observed in *Dab2$^{fl/fl}$:Cd4* mice involved FOXP3$^+$ Treg cells. In contrast, *Dab2$^{fl/fl}$:Cd2* mice

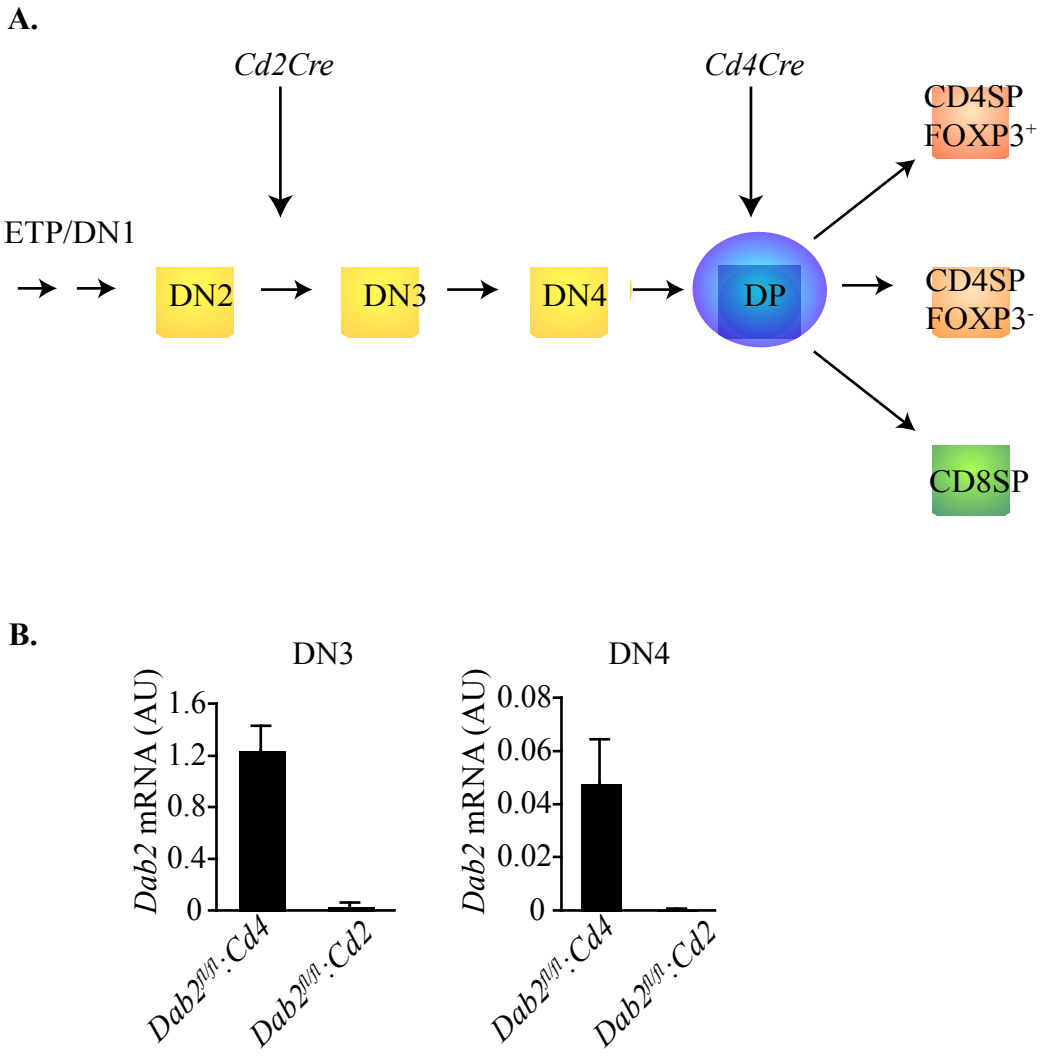

Figure III-5: Precursor stage specific deletion of *Dab2* using *Cd2CreTg*$^{+}$ and *Cd4CreTg*$^{+}$ mice

Figure III-5: Precursor stage specific deletion of *Dab2* using *Cd2CreTg*$^{+}$ and *Cd4CreTg*$^{+}$ mice.

A. Schematic of thymocyte development depicting the stages at which Cd2Cre and CD4Cre transgenes are expressed and delete flox'd target substrates. **B.** Real-time PCR analysis of *Dab2* expression in sorted DN3 (CD4-CD8-CD44-*CD25*$^{+}$) and DN4 (CD4-CD8-CD44-*CD2*5-) cells from *Dab2* $^{fl/fl}$*:Cd4* and *Dab2*$^{fl/fl}$*:Cd2* mice. Data are normalized to *Actb* mRNA and are representative of three independent experiments. **AU:** Arbitrary Units.

exhibited extensive alterations in T cell development and homeostasis, in addition to the common Treg cell defect.

We first confirmed that Dab2 expression was lost in the precursor cells of *Dab2*$^{fl/fl}$*:Cd2*, but not in *Dab2*$^{fl/fl}$*:Cd4* mice. Real-time PCR analysis of DN3 and DN4 thymocytes showed that the *Dab2* mRNA is absent in *Dab2*$^{fl/fl}$*:Cd2* compared to *Dab2*$^{fl/fl}$*:Cd4* mice. (**Fig. III-5B**). *Dab2* deletion at the thymic precursor stage resulted in subtle alterations in T cell development. The average total thymocyte number was consistently decreased by 50% in *Dab2*$^{fl/fl}$*:Cd2* mice relative to *Dab2*$^{fl/fl}$*:Cd4* or *wt* mice (**Fig. III-6A**), but the distribution of precursor and mature αβ T cells was relatively normal (**Fig. III-6B, C**). The absolute numbers of γδTCR^{+} was normal as their proportions among thymocytes increased (**Fig. III-6D**). However, one of the most notable effects of *Dab2* deletion at the precursor stage was on the frequency and number of NKT cells which was significantly decreased (**Fig. III-6D** and data not shown). The *Dab2* deficiency did not result in a significant increase in apoptosis of thymocyte subsets, as cell surface AnnexinV expression profiles of thymocytes from the CKO and LMC mice were similar (data not shown).

In the periphery, *Dab2* deficiency resulted in decreased cellularity in the spleen and lymph nodes of *Dab2*$^{fl/fl}$*:Cd2*, but not *Dab2*$^{fl/fl}$*:Cd4* mice (**Fig. III-7**). There was a significant decrease in the frequency of CD8^{+}, and to a lesser extent CD4^{+} T cells in secondary lymphoid organs of *Dab2*$^{fl/fl}$*:Cd2* mice (**Fig III-8A**). The deficit largely arose from the loss of naïve T cells, as the number of activated (CD44^{hi}CD62L^{lo}) T cells was comparable to LMCs (**Fig. III-8B**). The decrease in

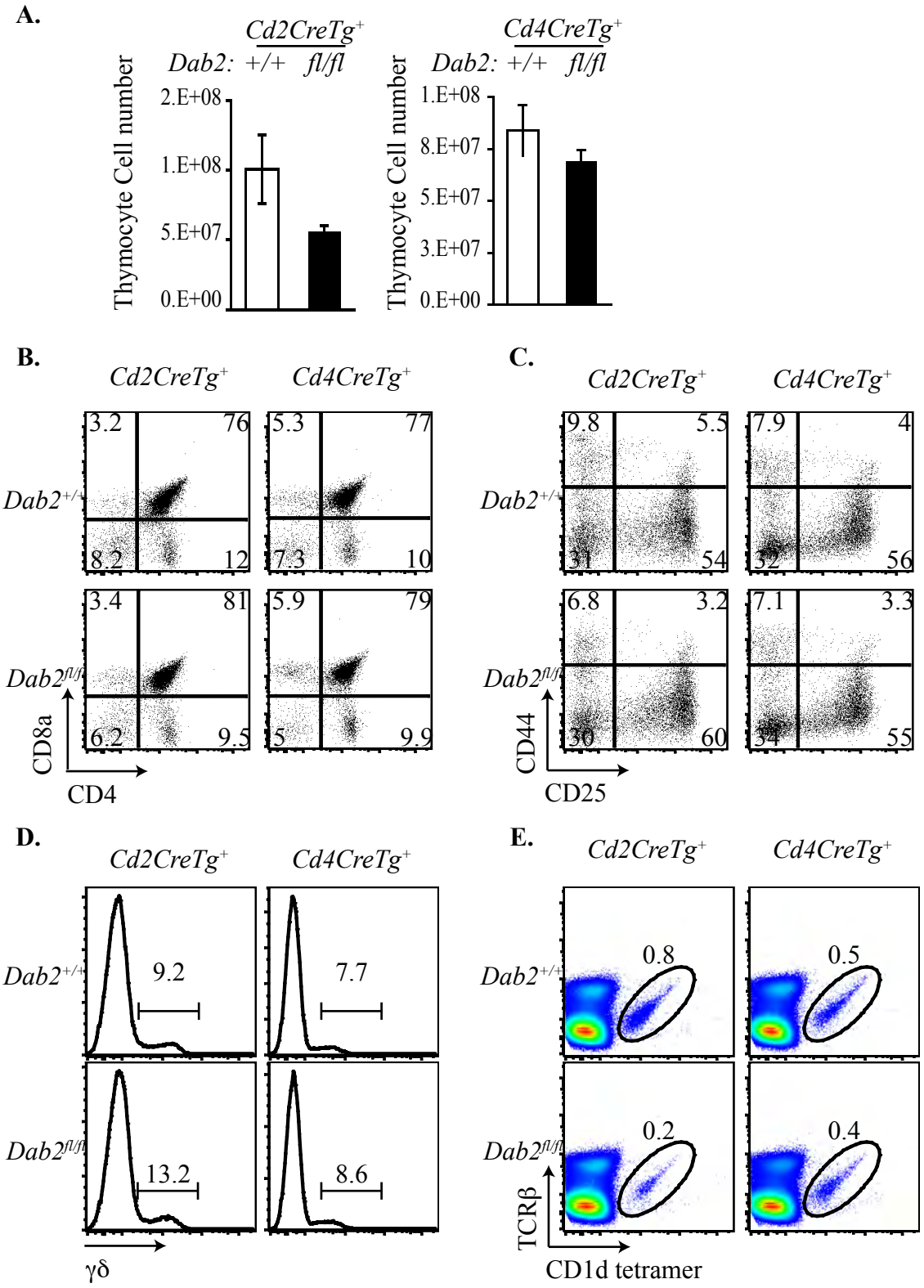

Figure III-6: Altered thymocyte development in *Dab2$^{fl/fl}$:Cd2* mice

Figure III-6: Altered thymocyte development in *Dab2$^{fl/fl}$:Cd2* mice

A. Total thymocyte numbers from 4-6 weeks old *wt, Dab2$^{fl/fl}$:Cd2* and *Dab2$^{fl/fl}$:Cd4* mice. **B.** Thymocytes from *wt, Dab2$^{fl/fl}$:Cd2* and *Dab2$^{fl/fl}$:Cd4* mice were analyzed for expression of CD4 and CD8 markers by flow cytometry. **C.** Thymocytes from *wt, Dab2$^{fl/fl}$:Cd2* and *Dab2$^{fl/fl}$:Cd4* mice were analyzed for expression of CD44 and CD25 markers on TN ($CD4^-CD8^-CD3^-$) cells by flow cytometry. **D.** Thymocytes from *wt, Dab2$^{fl/fl}$:Cd2* and *Dab2$^{fl/fl}$:Cd4* mice were analyzed for expression of the γδ TCR by flow cytometry. **E.** Thymocytes from *wt, Dab2$^{fl/fl}$:Cd2* and *Dab2$^{fl/fl}$:Cd4* mice were analyzed for expression of TCRβ and CD1d tetramer by flow cytometry to identify NKT cells.

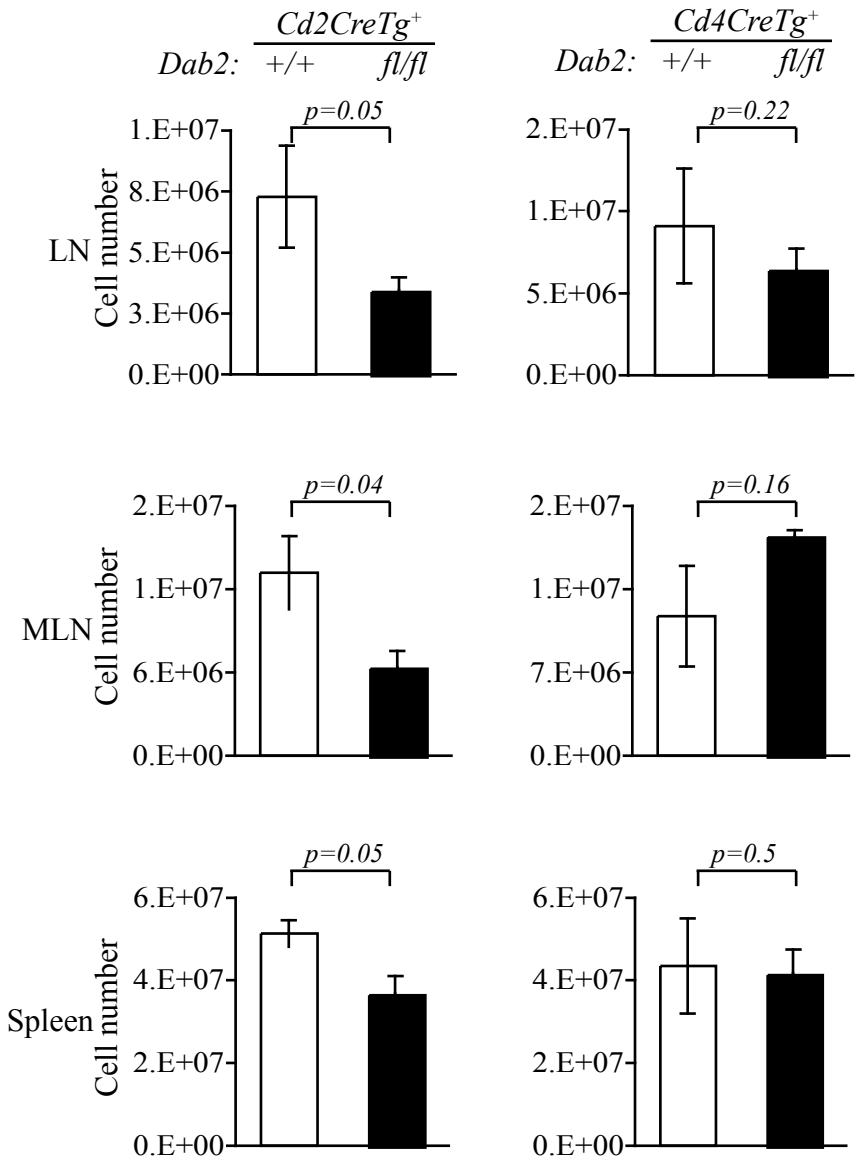

Figure III-7: *Dab2$^{fl/fl}$:Cd2* mice have decreased cellularity of secodnary lymphoid organs compared to *Dab2$^{fl/fl}$:Cd4* mice.

Figure III-7: *Dab2$^{fl/fl}$:Cd2* mice have decreased cellularity of secondary lymphoid organs compared to *Dab2$^{fl/fl}$:Cd4* mice.

Total lymphocyte cellularity in peripheral lymph nodes (LN: inguinal, brachial and axillary), mesenteric lymph node (MLN) and spleen of 6-8 weeks old *wt*, *Dab2$^{fl/fl}$:Cd4* and *Dab2$^{fl/fl}$:Cd2* mice. *p* values are shown on each graph.

peripheral lymphocyte number was not due to increased apoptosis of T cells in peripheral lymphoid organs, as measured by AnnexinV staining (data not shown).

Similar to *Dab2*$^{fl/fl}$*:Cd2* mice, *Cd4*Cre Tg^{+} *TGFβRII*$^{fl/fl}$ (*TGFβRII*$^{fl/fl}$: *Cd4*) mice also have reduced frequencies of CD8^{+} T cells in the thymus and spleen, in addition to reduced frequencies of NKT cells in these organs(264, 265). Consistent with the decrease in NKT cell numbers in the thymus of *Dab2*$^{fl/fl}$*:Cd2* mice, the frequency of NKT cells in the spleen was also decreased more than 2-fold, leading to a ~4 fold reduction in their numbers in the spleen (**Fig. III-8C**). However, this decrease was not seen in *Dab2*$^{fl/fl}$*:Cd4* CKO mice (**Fig. III-8C**). These results indicated that *Dab2* deficiency in thymic precursors, similar to the *TGFβRII*-deficiency in αβ T cells starting at the CD4^{+}CD8^{+} double positive thymocyte stage, led to the aberrant development and/or maintenance of naïve lymphocytes, CD8^{+} T and NKT cells. These results suggest that the developing T cells in *Dab2*$^{fl/fl}$*:Cd2* mice are likely impaired in TGFβ signaling. The defects are hematopoietic cell-intrinsic since in mixed bone marrow (BM) chimeras generated using irradiated *Rag1*$^{-/-}$ hosts reconstituted with 1:1 mixtures of T cell-depleted BM cells from *Dab2*$^{fl/fl}$*:Cd2* and *wt* mice the selective loss of NKT and CD8^{+} T cells was observed among donor cells originating from the Dab2-deficient T cell progenitors, but not among the partner *wt* cells. No alterations in T cell subset reconstitution were observed in *Dab2*$^{fl/fl}$*:Cd4 and wt* mixed BM chimeras (data not shown).

Altered TGFβ responsiveness of naïve conventional T cells from *Dab2*$^{fl/fl}$*:Cd2* mice.

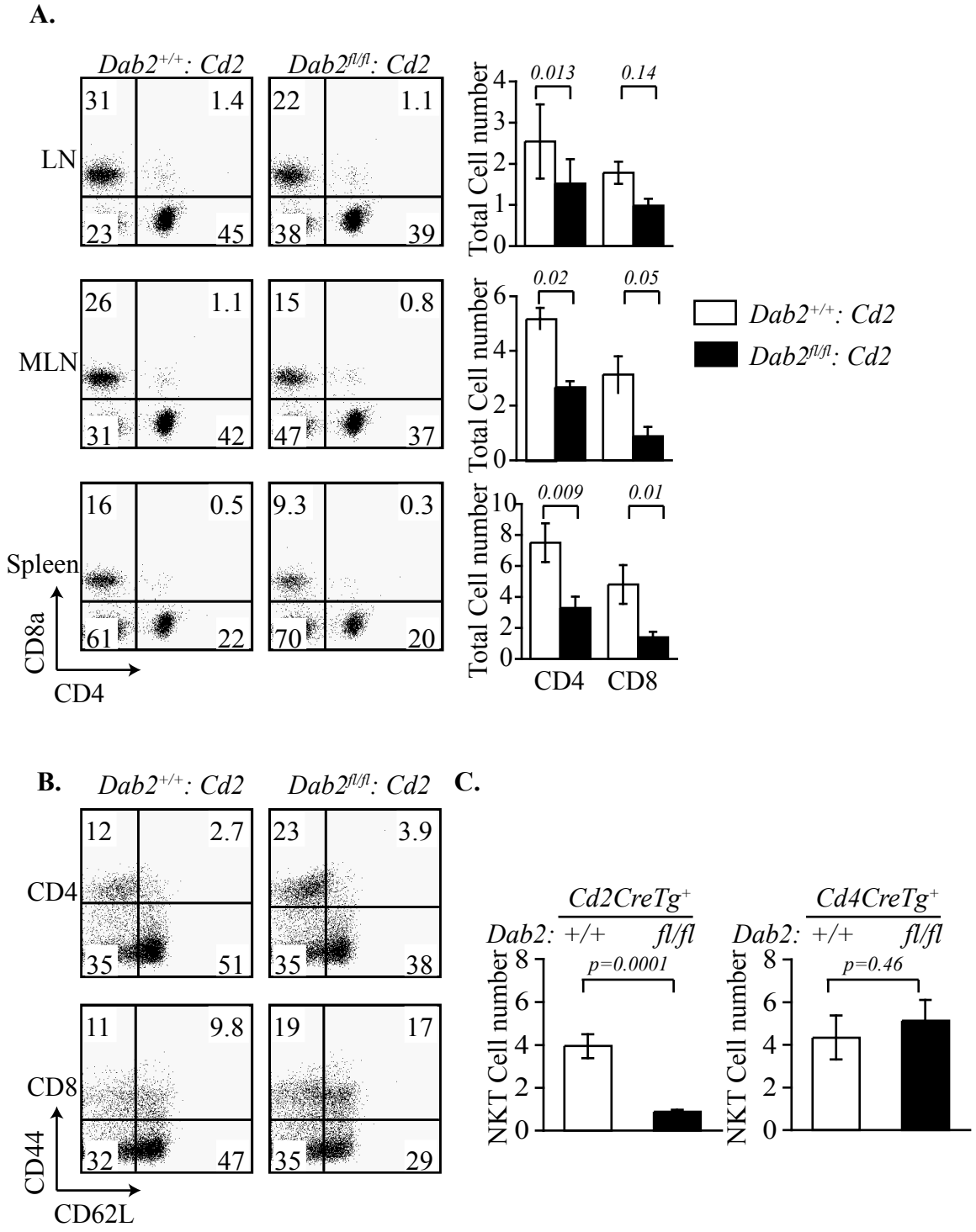

Figure III-8: Altered peripheral homeostasis of lymphocytes in *Dab2$^{fl/fl}$:Cd2* mice.

Figure III-8: Altered peripheral homeostasis of lymphocytes in *Dab2 $^{fl/fl}$:Cd2* mice.

A. *Left;* Representative flow cytometric dot plot showing frequency of CD4 and CD8 cells in LN, MLN and spleen of *wt* and *Dab2$^{fl/fl}$:Cd2* mice. *Right;* Numbers of $CD4^+$ and $CD8^+$ cells in LN, MLN and spleen. **B.** Flow cytometry analysis of expression of CD44 and CD62L on $CD4^+$ and $CD8^+$ T cells in the spleen of *wt* and *Dab2$^{fl/fl}$:Cd2* mice. **C.** Numbers of CD1d tetramer$^+$TCRβ^+ (NKT) cells in the spleen of *Dab2 $^{fl/fl}$:Cd4*, *Dab2$^{fl/fl}$:Cd2* and *wt LMC* mice. Data in (**A), (B), and (C)** are representative of more than five independent experiments with 2-3 mice per group. p values are indicated on each graph (error bars, SEM)

Given that conventional mature T cells do not express *Dab2*, and that *Dab2*$^{fl/fl}$*:Cd2* and *Dab2*$^{fl/fl}$*:Cd4* mice have similar alterations in Treg cells, the peripheral T cell defect in *Dab2*$^{fl/fl}$*:Cd2* mice is primarily due to the effect of *Dab2* deficiency originating from the precursors, and is independent of defects in Treg cells that are common to *Dab2*$^{fl/fl}$*:Cd2* and *Dab2*$^{fl/fl}$*:Cd4* mice nor can it prevented by *wt* Treg cells in the mixed BM chimeras. We reasoned that since DAB2 is an intermediate of the TGFβ signaling pathway shown to enhance SMAD2/3 activation (248), and *Dab2*$^{fl/fl}$*:Cd2* mice exhibit defects in lymphocyte subsets overlapping with those observed in *TGFβRII*$^{fl/fl}$:CD4 mice, the TGFβ responsiveness of conventional CD4^{+}CD25^{-} T cells from *Dab2*$^{fl/fl}$*:Cd2*, but not Dab2$^{fl/fl}$:CD4, mice may be altered. To test this we first examined whether the proliferation of naïve T cells from *Dab2*$^{fl/fl}$*:Cd2* mice was inhibited by TGFβ *in vitro*. CFSE-labeled naïve CD4^{+}CD25^{-} conventional T cells from *wt*, *Dab2*$^{fl/fl}$*:Cd2* and *Dab2*$^{fl/fl}$*:Cd4* mice were activated with anti-CD3 and anti-CD28, with or without TGFβ. While T cells from *wt* and *Dab2*$^{fl/fl}$*:Cd4* mice were inhibited by TGFβ in the culture (***Fig. III-9A***), that resulted in decreased cell recovery (data not shown), proliferation of T cells from *Dab2*$^{fl/fl}$*:Cd2* mice was not affected by the addition of TGFβ.

TGFβ plays a pivotal role in regulating the balance between the generation of FOXP3^{+} Treg cells and pro-inflammatory Th17 cells (285, 286). TGFβ can direct the conversion of conventional murine CD4^{+} T cells into Foxp3 expressing anti-inflammatory Treg cells *in vitro*, but in the presence of proinflammatory cytokine

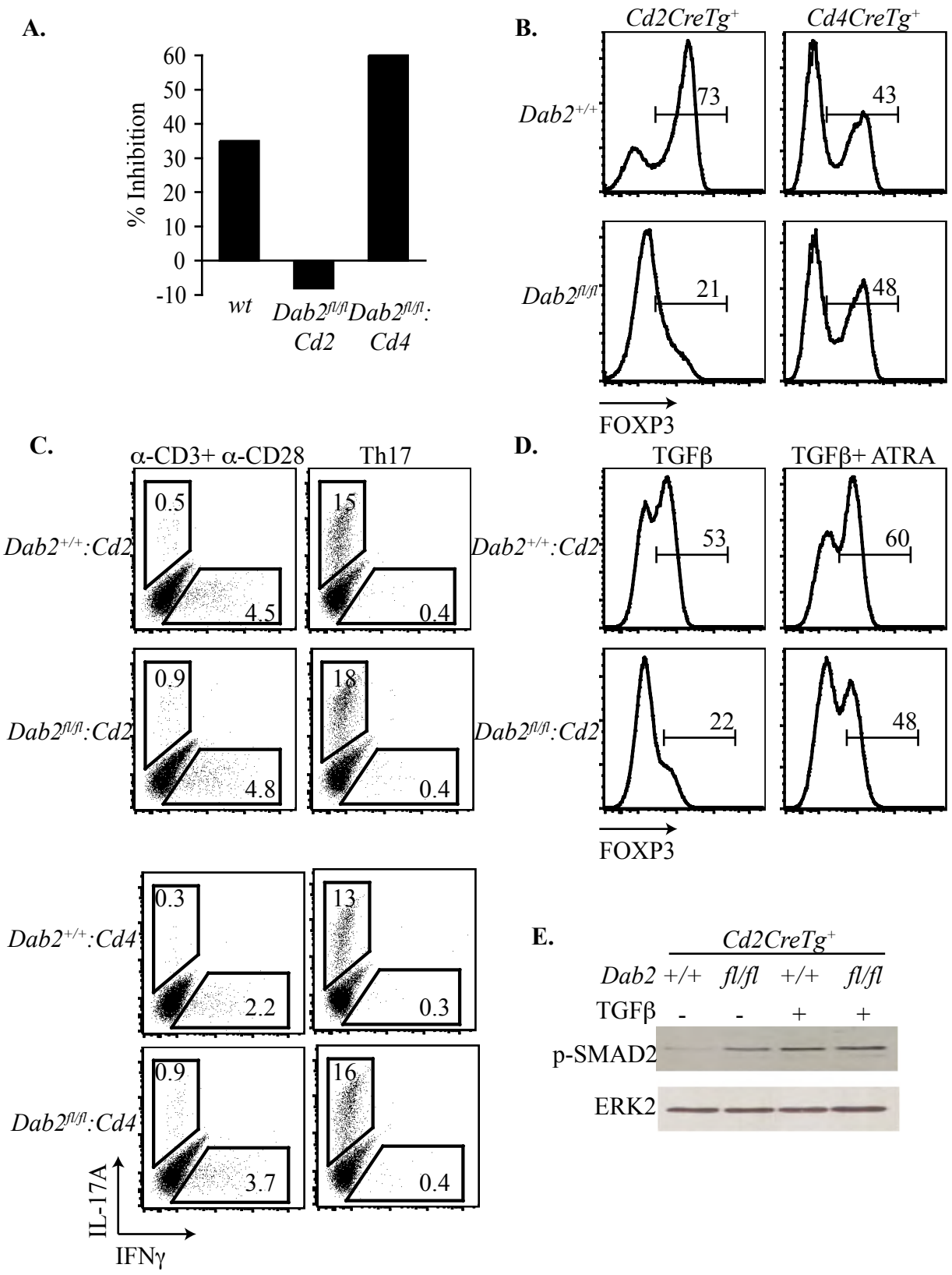

Figure III-9: Altered TGFβ responsiveness of peripheral CD4$^+$CD25$^-$ cells from *Dab2$^{fl/fl}$:Cd2* mice

Figure III-9: Altered TGFβ responsiveness of peripheral $CD4^+CD25^-$ cells from *Dab2$^{fl/fl}$:Cd2* mice.

A. Sorted and CFSE loaded $CD4^+CD25^-$ conventional T cells from *wt*, *Dab2$^{fl/fl}$:Cd4* and *Dab2$^{fl/fl}$:Cd2* mice were activated with anti-CD3 plus anti-CD28 in the presence or absence of rTGFβ (2ng/ml). Percent inhibition of proliferation by TGFβ was calculated using the formula ((%Divided-$_{TGFβ}$ - %Divided$^+_{TGFβ}$)/ %Divided-$_{TGFβ}$)*100. One of three independent experiments is shown. **B.** Representative flow cytometric analysis of induced FOXP3 expression in sorted $CD4^+CD25^-$ T cells from *Dab2 $^{fl/fl}$:Cd4*, *Dab2$^{fl/fl}$:Cd2* and *wt* LMC mice stimulated *in vitro* with TGFβ. One of five independent experiments is shown. **C.** Flow cytometric analysis of IL-17 and IFNγ production by TGFβ and IL-6 stimulated $CD4^+CD25^-$ T cells from *Dab2 $^{fl/fl}$:Cd4*, *Dab2$^{fl/fl}$:Cd2* mice and *wt* LMC mice. Cells were also stimulated under non-skewing conditions to establish baseline cytokine production. One of five independent experiments with similar results is shown. **D.** Flow cytometric analysis of FOXP3 expression in TGFβ and ATRA stimulated $CD4^+CD25^-$ cells from *wt* and *Dab2$^{fl/fl}$:Cd2* mice. **E.** Western blot analysis of SMAD2 phosphorylation in total splenocytes from *wt* and *Dab2$^{fl/fl}$:Cd2* mice stimulated with (+) and without (-) TGFβ for 1 hr at 37^{o}C; ERK2, loading control.

such as IL-6, TGFβ can divert naïve $CD4^+$ T cells to the Th17 lineage. We compared the ability of $CD4^+CD25^-$ T cells from *Dab2*$^{fl/fl}$*:Cd2*, *Dab2*$^{fl/fl}$*:Cd4*, and LMC mice to differentiate into these two lineages. $CD4^+CD25^-$ T cells from *Dab2*$^{fl/fl}$*:Cd2* mice were inefficient at generating $FOXP3^+$Treg cells when cultured TGFβ, while T cells from *Dab2*$^{fl/fl}$*:Cd4* mice were similarly responsive as *wt* T cells to TGFβ to induce FOXP3 expression ***(*Fig. III-9B*)***. In contrast, $CD4^+$ T cells from *Dab2*$^{fl/fl}$*:Cd2* mice were efficient in generating IL-17 producers when TGFβ and IL-6 were present in culture ***(*Fig. III-9C*).*** There was also a small but consistent increase in the frequency of Th17 cells generated by *Dab2*$^{fl/fl}$*:Cd2* $CD4^+$T cells as compared to *Dab2*$^{fl/fl}$*:Cd4* $CD4^+$T cells. This indicated that the TGFβ signaling in T cells from *Dab2*$^{fl/fl}$*:Cd2* mice was inadequate to induce FOXP3 expression, but it was sufficient to differentiate naïve $CD4^+$ T cells to the Th17 lineage and that the signaling pathway required for the inhibition of FOXP3 expression by proinflammatory cytokine IL-6 was also intact. The *Dab2*-deficient T cells were not generally altered in $CD4^+$ T cell subset differentiation since they were normal in their ability to generate Th1 cells in a Th1-skewing culture condition, and further, TGFβ signals required to inhibit Th1 differentiation were also intact (data not shown). These results point to a specific defect in TGFβ signaling to induce *Foxp3* expression in $CD4^+$ T cells from *Dab2*$^{fl/fl}$*:Cd2* mice that had developed from precursors lacking *Dab2*.

The vitamin A metabolite ATRA has also been implicated in maintaining a balance between the generation of regulatory versus inflammatory T cell subsets by synergizing with TGFβ to block proinflammatory cytokine driven Th17 subset generation (296). While ATRA cannot induce *Dab2* expression in the absence of

TGFβ in naïve *wt* T cells (**Fig. III-1D**), it was possible that ATRA could enhance and/or bypass TGFβ signals leading to induction of FOXP3 that were altered in naïve $CD4^+$ T cells from *Dab2*$^{fl/fl}$*:Cd2* mice. Indeed, the addition of ATRA along with TGFβ was able to overcome the aberrant TGFβ signaling in *Dab2*-deficient T cells to produce a normal proportion of $FOXP3^+$ T cells (**Fig. III-9D**). Hence, the defect in T cells generated from *Dab2*-deficient precursors is due to impairment in TGFβ signaling, one which can be overcome by synergistic ATRA signals. These results also demonstrate that the inducible *Dab2* expression in conventional T cells is not non-redundantly required for ATRA-mediated promotion of $FOXP3^+$ T cell production.

Finally, since *Dab2*$^{fl/fl}$*:Cd2* conventional T cells were impaired in TGFβ signaling, we investigated the activity of TGFβ-activated nuclear effectors SMAD2 and SMAD3 by examining their phosphorylation state in TGFβ treated T cell cultures. While *wt* $CD4^+$ T cells cultured without TGFβ contained minimal amounts of phosphorylated SMAD2 protein, the basal level of SMAD2 (and SMAD3, data not shown) phosphorylation was higher in *ex vivo* T cells and $CD4^+$ thymocytes from *Dab2*$^{fl/fl}$*:Cd2* mice (**Fig. III-9E** and data not shown). Within 30-60 min of TGFβ stimulation maximally elevated amounts of SMAD2 phosphorylation was detectable in $CD4^+$ T cells from *wt* mice, but in the mutant T cells minimal TGFβ-dependent induction was observed. SMAD3 phosphorylation pattern in $CD4^+$ T cells from *Dab2*$^{fl/fl}$*:Cd4* mice was comparable to those from LMCs (data not shown). These results indicate that the regulation of the receptor associated SMAD2/3 activation is aberrant in T cells that had differentiated from *Dab2*-deficient precursors.

Discussion

Dab2 is a FOXP3 target gene whose expression among peripheral lymphocytes is restricted to $CD4^+$ Treg cells. We have shown that *Dab2* is absolutely required for *in vitro* and *in vivo* suppression of effector T cells by Treg cells. Strikingly, DAB2 also has a unique, FOXP3-independent function in ensuring normal development and maintenance of TGFβ-dependent lymphocyte subsets and programming TGFβ responsiveness of conventional ab lineage T cells. By virtue of the restricted *Dab2* expression in T cell precursors and the defects associated with its absence, it is apparent that the TGFβ signaling competence of mature T cells is set during their early intrathymic differentiation. The absence of *Dab2* in thymic precursor cells results in impaired persistence of TGFβ-dependent $CD8^+$ T cells and NKT cells in the periphery and the generation of mature conventional $CD4^+$ T cells that are defective in TGFβ signaling. We propose that DAB2 is necessary to calibrate TGFβ signaling during early T cell development since the impairment in TGFβ signaling is selective and appears to affect the processes requiring relatively strong TGFβ signaling, such as the induction of $FOXP3^+$ T cells from naïve T cells (273). Further, the defect can be overcome by ATRA, a factor that normally enhances TGFβ signaling in T cells, indicating that the absence of DAB2-mediated calibration in the thymic precursors does not disable TGFβ signaling completely.

Given the FOXP3-dependent expression of *Dab2* among mature lymphocytes and the critical function of *Dab2* in setting TGFβ responsiveness during T cell development, one prediction was that *Dab2* CKO mice would phenocopy the loss of

Treg cells observed in mice with defective TGFβ signaling (Rubtsov and Rudensky, 2007). However, *Dab2* CKO mice maintained normal numbers of Treg cells, indicating that TGFβ signaling requirements are distinct in conventional T cells versus Treg cells. Further, *Dab2* CKO mice are healthy suggesting that *Dab2*-deficient Treg cells are functionally intact *in vivo*. Curiously, defects in *Dab2*-deficient Treg cells are revealed *in vivo* only when these cells are purified and transplanted along with effector T cells into immunodefficient $Rag^{-/-}$ mice. In vitro, however, *Dab2*-deficient Treg cells cannot suppress conventional T cell proliferation. This dichotomy suggests that either the *in vitro* suppression relies on a very different mechanism than the *in vivo* Treg cell activities and/or that the latter involves multiple, possibly redundant, modes of immunosuppression. Data exist to support both possibilities. For instance, IL-10, TGFβ and CTLA-4 are not required for Treg cell-mediated suppression of effector T cells *in vitro* (316), but each is variably required in different *in vivo* settings of immune suppression by Treg cells (130, 221, 235). None of these effectors appear to be aberrantly regulated in *Dab2*-deficient Treg cells. Conversely, while IL-35 is necessary for optimal Treg cell function *in vitro*, un-manipulated $Il35^{-/-}$ mice exhibit normal T cell homeostasis (135). *Dab2*-deficient Treg cells express comparable amounts of FOXP3-regulated *Ebi3* at the RNA level and they are functionally distinct from $Ebi3^{-/-}$ Treg cells, suggesting that IL-35 and DAB2 belong to two independent regulatory pathways. Although the reduced GAP junction activities appear to be the only distinguishing feature of *Dab2*-deficient Treg cells that has previously been correlated with the impaired *in vitro* immune suppression, whether this is the cause of the *Dab2*-deficient Treg cell defect remains to be proven.

Irrespective of the underlying mechanism of *in vitro* suppression, the relevance of DAB2 for Treg function *in vivo* remains to be determined. There are at least three non-mutually exclusive explanations to account for the maintenance of T cell homeostasis in un-manipulated Dab2 CKO mice: One, biochemical processes regulated by DAB2 is not essential for *in vivo* Treg function. Two, DAB2-mediated function may only be critical in settings other than in homeostasis, such as during infection when relative TGFβ availability may be limiting. Three, other compensatory components can mask the effects of *Dab2*-deficiency, especially a myriad of Treg cell-intrinsic and cell-extrinsic TGFβ signaling modulators that have yet to be characterized in the lymphoid system. One appealing candidate for the latter is the ATRA signaling, which appears to be operational in Dab2-deficient T cells (**Fig. III-9D**), and one that has been shown to synergize with TGFβ for enhancing Treg cell generation from naïve $CD4^+$ T cells at the expense of pro-inflammatory Th17 development. ATRA can override the TGFβ signaling deficiencies in naïve $CD4^+$ T cells generated from *Dab2*-deficient precursors to induce $FOXP3^+$ T cells *in vitro*, and the normal function of *Dab2*-deficient Treg cells *in vivo* may also result from ATRA or other synergistic measures available to compensate for the intrinsic, suboptimal TGFβ or other DAB2-regulated activities in T cell subsets. The possibility that ATRA is masking the effects of *Dab2*-deficiency is currently being examined in Vitamin A and *Dab2* double deficient mice.

One of the unexpected observations about DAB2 function is its involvement during early T cell development in calibrating TGFβ signaling competence of the mature T cell progenies. The TGFβ signaling defect observed in $Dab2^{fl/fl}$*:Cd2* mice

only partly recapitulates the immune dysregulation in *TGFβRII*$^{fl/fl}$:CD4 mice (238, 264, 265). While the loss of $CD8^+$ T, NKT and naïve T cells and increased production of Th1 cytokine IFNγ (data not shown) are observed in both mouse mutant models, *TGFβRII*$^{fl/fl}$:CD4 mice are replete with activated T cells and they succumb to lethal autoimmune diseases that cannot be completely reversed by the infusion of *wt* Treg cells (264). An explanation for this difference is that the *Dab2*-deficiency alters the quality and perhaps the threshold of TGFβ signaling but it does not abrogate the TGFβ function in all cell types so that the generation and maintenance of TGFβ-regulated T cell subsets such as Treg and Th1 cells *in vivo* and Treg cell-independent, TGFβ signaling-dependent maintenance of naïve T cell quiescence are relatively unperturbed.

The biochemical basis for the altered TGFβ signaling capacity originating in the T cell precursors of *Dab2*$^{fl/fl}$*:Cd2* mice is not addressed here, but it appears that the receptor-associated SMADs can be phosphorylated in *Dab2*-deficient T cells, suggesting that the defect lies downstream of the membrane proximal events. Although the increased amount of activated SMAD2/3 in non-TGFβ treated, *ex vivo* T cells from *Dab2*$^{fl/fl}$*:Cd2* mice (***Fig. 4E***) can be viewed as evidence for DAB2 as a negative regulator of TGFβ signaling, T cells that had developed from *Dab2*-deficient precursors are clearly not hyper-responsive to TGFβ *in vivo* or *in vitro*. In some cancer cells that are insensitive to TGFβ-mediated growth inhibition, hyperactivation of SMAD2/3 is also observed, indicating that enhanced basal SMAD2/3 phosphorylation is not necessarily linked to enhanced TGFβ-responsiveness (335). Rather, the aberrant pattern of SMAD2/3 phopsphorylation in conventional T cells of

Dab2$^{fl/fl}$*:Cd2* mice may indicate an adaptation by the TGFβ signaling defective T cells to be maintained in the periphery or a selective enrichment of the "adapted" T cells. Given the association of DAB2 with diverse intracellular processes in non-lymphoid cells, it is currently a major challenge to determine the repertoire of DAB2 activities in T cells and to characterize additional components of the TGFβ signaling cascade that may interact with DAB2. Hence, although convincing data exist to indicate that DAB2 positively controls TGFβ signaling during development and in diverse cell types (248, 249, 318, 323, 324), it remains formally possible that in T cells the apparent defects in TGFβ signaling may arise from alterations in other Dab2-dependent pathways that can impact TGFβ signaling competence. Single cell tracking of *Dab2* expression in the differentiating precursors and detailed biochemical studies of TGFβ signaling in the absence of *Dab2* are required to definitively establish how *Dab2* programs TGFβ signaling competence of mature T cells during their early intrathymic differentiation.

Collectively, DAB2 is modeled as a cell-intrinsic modulator of TGFβ signaling during T cell differentiation and selective expression of *Dab2* in discreet cell subsets may permit cell lineage diversification based on the differential sensing of TGFβ. Consistent with the strength of TGFBβ signaling as a decisive event in T cell differentiation it has been shown recently that low concentrations of TGFβ synergize with IL-6 to drive pro-inflammatory Th17 generation from naïve $CD4^+$ T cells while high concentrations of TGFβ program anti-inflammatory Treg cell generation (273). The restricted FOXP3-regulated DAB2 expression in Treg cells may contribute to amplified, strong TGFβ signaling in a positive auto feedback loop. Additionally,

naïve $CD4^+$ T cells that had differentiated from *Dab2*-deficient precursors favor Th17 generation at the expense of $FOXP3^+$ Treg cells because they have reduced sensitivity to TGFβ. Importantly, ATRA can restore TGFβ signaling capacity of naïve $CD4^+$ T cells generated from Dab2-deficient precursors, emphasizing the cooperative nature of these two signaling pathways in promoting Treg cell development and maintenance.

Materials and Methods

Mice

Dab2$^{fl/fl}$ (B6 x 129) mice (318) were backcrossed 4 times onto B6 background. T cell specific deletion of *Dab2* was obtained by crossing *Dab2*$^{fl/fl}$ mice with *Cd2*$CreTg^+$ (317) and *Cd4*$CreTg^+$ mice (336). Genotypes were determined by PCR analysis. Mice were analyzed at 6-8 weeks of age unless otherwise indicated. *Foxp3*eGFP reporter mice (285) were a kind gift from V. Kuchroo, provided by L. Selin. All mice used in these experiments were housed in a specific pathogen-free rodent barrier facility. All experiments were approved by the University of Massachusetts Medical School Institutional Care and Use Committee.

Antibodies, Flow cytometry and cell sorting

Fluorescently labeled antibodies specific to CD4, CD8a, NK1.1, CD44, CD62L, CTLA-4, CD103, IL-17, IFN-g and Granzyme B were purchased from BD Pharmingen and antibodies specific to Foxp3, CD25, CD127, GITR, CD3e and TCRb were purchased from eBiosciences. Intra-cellular staining for IL-17, IFN-g and Foxp3 were performed according to the manufacturer's protocol. PE conjugated a-gal-

ceramide tetramer was a kind gift from L. Berg (UMMS). All samples were acquired on an EPICS XL cytometer (BD-Coulter, Hialeah, FL) or LSRII (BD), and data were analyzed using FlowJo software (Treestar, San Carlos, CA). *Ex vivo* thymic subsets, peripheral T cell subsets including $CD4^+CD25^-$ conventional T cells and $CD4^+CD25^+$ Treg cells were sorted to greater than 95% purity using MoFlo (Cytomation) cell sorter.

Retroviral Infection

NFC ($CD4^+CD8^+$, DP) thymoma cells were infected with a control MSCV retroviral vector containing GFP or with an MSCV retroviral vector carrying the full-length Foxp3cDNA cloned upstream of an IRES and GFP. Stable cell lines that express vector alone (V) and MSCV-Foxp3 (F) were generated by cell sorting and maintained in complete DMEM.

RT-PCR and real-time PCR

For reverse transciption-polymerase chain reaction (RT-PCR), total RNA was isolated from purified cells (Trizol Reagent, Invitrogen) and cDNA was prepared using an Omniscript RT-PCR kit (Qiagen). For semi-quantitative RT-PCR (sqRT-PCR), 5-fold serial dilutions of cDNA were used. PCR primers used are listed in **Supplemental Data 1**. For real-time PCR, cDNA was prepared as described, and amplification was performed using iQ SYBR Green supermix (BioRad). All data were normalized to *Actb* mRNA expression and represented as arbitrary units (AU).

Chromatin Immunoprecipitation Assays

Foxp3 over-expressing NFC cells (F) and vector control cells (V) were stimulated with PMA (50ngml) and Ionomycin (100ng/ml) at 37^{o}C for 24 hours. ChIP assays were performed on 1 x 10^6 cells using the ChIP Assay Kit (Upstate Cell signaling Solutions, Charlottesville, VA). Immuno-precipitation was performed using anti-FOXP3 antibody (Santa Cruz). The recovered DNA was dissolved in 20 ml of H_2O and analyzed by PCR. PCR primers used were: *Utr2*-for 5'- GTA CAG TAA TTG GAT AGA CTT TCC-3' and *Utr2*-rev 5' GAG CAA GAG CTC CTG GCA GGC-3'; *Utr1*-for 5'-AGG CAA AGA ATA GTA TAA AAT TCT-3' and *Utr1*-rev 5'- TCC AGC AAG CAA GGG AAG GTC ATT-3'; *Int1.1*-for 5'-CTG TGT ATC ATA TAT TTA CAT TAT-3' and *Int1.1*-rev 5'- AGT GTT TCT CAA TCT CCC CAA TGC-3'; *Int1.2*-for 5'-CAT GAG TTC TGG AGA ACA AAA TTC-3' and *Int1.2*-rev 5'-TGC TTG TGG ATA CAC GTA TCA AAG-3'; *Pde3b-20*-for 5-TTT GGG CCG CAT AGA GAA AA-3' and *Pde3b-20*-rev 5'-CAG TGA ATC AGC AGC ACA A-3'.

In vitro suppression assay

Sorted CD4$^+$*CD25*$^+$ were activated *in vitro* with plate-bound anti-CD3 (0.5mg/ml; clone 500A2) and anti-*CD2*8 (1.0mg/ml; clone 37N) in the presence of 100U/ml of rIL-2 in complete DMEM (10% FBS, 50uM 2-ME, 2mM L-glutamine, 20mM Hepes, 0.1mM Non-Essential Amino Acids) for 48 hours. Post-stimulation, varying numbers of Treg cells were co-cultured with 5 x 10^4 freshly isolated CD4$^+$T cells and 5 x 10^4 irradiated splenocytes from B6 mice and activated with plate-bound anti-CD3 for 72 hours. Cultures were pulsed with 1uCi ^{3}H thymidine per well for the final 10-12 hours of culture and thymidine incorporation was analyzed on a scintillation counter.

Calcein AM intercellular transfer and cAMP ELISA

Sorted $CD4^+CD25^+$ Treg cells were loaded with Calcein as described in(144). Briefly, Treg cells (10^7/ml) were incubated with 1mM calcein AM at 37°C for 30 minutes in serum-free IMDM. After washing twice with IMDM, Tregs were co-cultured with $Ly5.1^+CD4^+$T cells and activated with plate-bound anti-CD3 (1mg/ml) and anti-CD28 (2mg/ml) for 16 hours. Calcein transfer was detected on an LSRII (BD). To measure intracellular cAMP amounts sorted $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were washed in ice-cold PBS and lysed in 0.1NHCl and a cAMP specific ELISA was performed according to the manufacturer's protocol (BioMol and Promega).

In vitro T cell activation and proliferation

Sorted $CD4^+CD25^-$ T cells were activated with plate-bound anti-CD3 (0.5mg/ml) and anti-CD28 (1.0mg/ml) in complete DMEM for 72 hours. For proliferation assays, 10^7cells/ml were labeled with 1mM CFSE (carboxyfluorescein diacetate succinimidyl diester; Molecular Probes) in PBS at 37°C for 15 minutes and then washed twice in complete DMEM. Proliferation was measured by analysis of the dilution of CFSE by flow cytometry. The following cytokines and antibodies were added for effector T cell skewing; *Foxp3 induction*: rTGFβ (2ng/ml; R&D), all-trans retinoic acid (ATRA) (100nM; Sigma); *Th17*: rTGFβ (2ng/ml), rIL-6 (20ng/ml; R&D), anti-IFNγ (10mg/ml; eBiosciences); *Th1*: rIL-12 (5ng/ml; R&D), anti-IL-4 (10mg/ml; BD Pharmingen).

Western Blot

$CD4^+$ T cells or thymocytes from *wt, Dab2$^{fl/fl}$:Cd4* and *Dab2$^{fl/fl}$:Cd2* mice were stimulated as described in the presence or absence of rTGFβ (2ng/ml) for 30 minutes at 37^{o}C. Cells were centrifuged and resuspended in lysis buffer (50mM Tris, pH7.5, 300mM NaCl, 5mM EDTA, 1%NP-40, 0.1mM Na_3VO_4 and 1x protease inhibitor cocktail (Roche)). Lysates were resolved by SDS-PAGE and electroblotted onto nitrocellulose membranes using a BioRad semi-dry transblot apparatus. Western blot was performed using antibodies to phospho-Smad-2 (Chemicon International), phospho-Smad3 and ERK1/2 (Cell Signaling Technology). Blots were revealed with isotype-specific HRP-conjugated secondary antibodies (Sigma) followed by enhanced chemiluminescence detection (Pierce, Rockford, IL).

Colitis induction and cure

4 x 10^5 $CD4^+CD25^-$ cells from *wt* mice were adoptively transferred to lymphocyte-deficient (*Rag1$^{-/-}$*) mice to induce colitis. To test the function of Treg cells in preventing the colitis, 2 x 10^5 $CD4^+CD25^+$ regulatory T cells from *wt, Dab2$^{fl/fl}$:Cd4* and *Dab2$^{fl/fl}$:Cd2* mice were co-injected. Mice were weighed weekly and examined for signs of colitis and wasting. For colitis "rescue" experiments, 1x10^6 $CD4^+CD25^+$ regulatory T cells from *wt* or *Dab2$^{fl/fl}$:Cd2* mice were injected into *Rag1$^{-/-}$* mice that had previously received 4x10^5 $CD4^+$CD25- T cells. Mice were euthanized if >25% weight loss was observed.

CHAPTER IV

GENERAL DISCUSSION

The immune system has evolved several ways to keep auto-reactive T cells in check. Central tolerance operating in the thymus is one mechanism of deleting self-reactive T cells. However, it is insufficient and additional mechanisms are required to ensure the integrity of host tissue and prevent autoimmunity in the periphery. Treg cells are critical in this aspect and dominant suppression of autoreactivity by these cells ensures peripheral T cell tolerance and immune homeostasis. This thesis elaborates the role of two mediators of Treg cell function: CTLA-4, a negative costimulatory molecule and DAB2, a TGFβ signaling intermediate.

It is widely accepted that CTLA-4 is an important negative regulator of T cell autoreactivity. *Ctla4*$^{-/-}$ mice display an aggressive autoimmune phenotype and die by 3-4 weeks of age. However, the underlying defect for this dramatic loss in peripheral tolerance is unclear and it is still not known when CTLA-4 functions during T cell activation, whether it has distinct functions in T cell subsets, and what its functional repertoire is. Manipulation of CTLA-4 expression *in vivo* has been difficult. Because CTLA-4 is expressed on both Treg cells as well as activated conventional T cells, the effects of anti-CTLA-4 antibody treatment *in vivo* are complicated to interpret. However, anti-CTLA-4 antibodies are currently being tested to treat melanoma and prostrate cancer and are poised to come to clinics in the next few years. Although effective, the treatment is associated with severe side effects that include colitis and uveitis. Paradoxically, tumor regression occurs despite increased Treg cell numbers. CTLA-4 is constitutively expressed on Treg cells, and data from our lab and others

have clearly shown that CTLA-4 on Treg cells is critical to prevent autoimmunity. Further, a recent study has suggested that blocking CTLA-4 on Treg cells by anti-CTLA-4 treatment *in vivo* increases their proliferation but at the same time makes them less potent at suppression (337). Accordingly, the original idea of using anti-CTLA-4 antibodies to remove the 'block' in T cell activation needs to be revised, and the cellular targets of anti-CTLA-4 antibody *in vivo* and their function remains to be identified.

Another approach has been to use RNAi mediated down-modulation of *Ctla4* transcripts in T cells *in vivo*. This results in an accelerated onset of diabetes in NOD mice as well as in NOD x B6.H-2g7 F1 mice that are normally resistant to diabetes. This phenotype is starkly different from that of *Ctla4*$^{-/-}$ mice that suffer from indiscriminate autoimmunity. Although the precise expression pattern of residual CTLA-4 expression in specific T cell subsets was not examined in this model, these results suggest that subtle modulation of CTLA-4 expression in T cells can yield distinct immunological outcomes. Indeed, differential levels of expression of CTLA-4 protein (and isoforms) resulting from *Ctla4* gene polymorphsims has been linked to susceptibility to several autoimmune diseases such as systemic lupus erythematosus (SLE), myasthenia gravis, Grave's disease and Type 1 Diabetes.

The *Ctla4*$^{-/-}$ mouse stands witness to the fact that CTLA-4 is important for preventing self-reactivity of T cells- however it is not a very useful model to understand the mechanism of CTLA-4 control over peripheral tolerance, primarily because of the aggressive disease that begins very early in age. Viable mouse models that allow temporal and spatial control of CTLA-4 expression are required to determine the precise regulation of T cell reactivity by CTLA-4. To begin to address

this issue, we have generated different mouse models in which CTLA-4 expression is restricted to distinct T cell subsets. Analyses of these mice reveal that CTLA-4 has dual and distinct function in activated T cells and Treg cells to maintain T cell homeostasis. We propose a model of CTLA-4 function in which CTLA-4 expression on FOXP3^{+} Treg cells is critical to prevent naïve conventional T cell activation in trans (**Figure IV-1**). In the absence of CTLA-4 expression on Treg cells, Tconv cells become aberrantly activated and proliferate. However, CTLA-4 expression on these activated T cells can moderate inflammation and autoimmune migration of these cells into tissues. Although this thesis does not directly address the mechanism by which CTLA-4 on Treg cells prevents autoimmunity of conventional T cells, we propose that one way is by modulation of antigen presenting cell (APC) function. In this model, CTLA-4 expressing Treg cells would efficiently engage B7 molecules on APCs and inhibit their capacity to activate Tconv cells. In the event of activation of Tconv cells to self-antigen, CTLA-4 expression on activated T cells would inhibit emigration into tissues most likely by altering expression of chemokine receptors on T cells. In preliminary experiments, we find that the expression of CCR7, a receptor essential for lymphocyte retention in lymph nodes and for egress from non-lymphoid tissues, is maintained on activated T cells that express CTLA-4 but not on *Ctla4*$^{-/-}$ T cells. This aspect of CTLA-4 function in regulating movement of activated T cells requires further investigation.

It is important to note here that all the data described in this study address CTLA-4 function in the context of 'aberrant' activation to self. Thus, during antigenic stimulation that is associated with inflammation, as in the case of an infection, for instance, CTLA-4 function may be very distinct and may involve regulation of

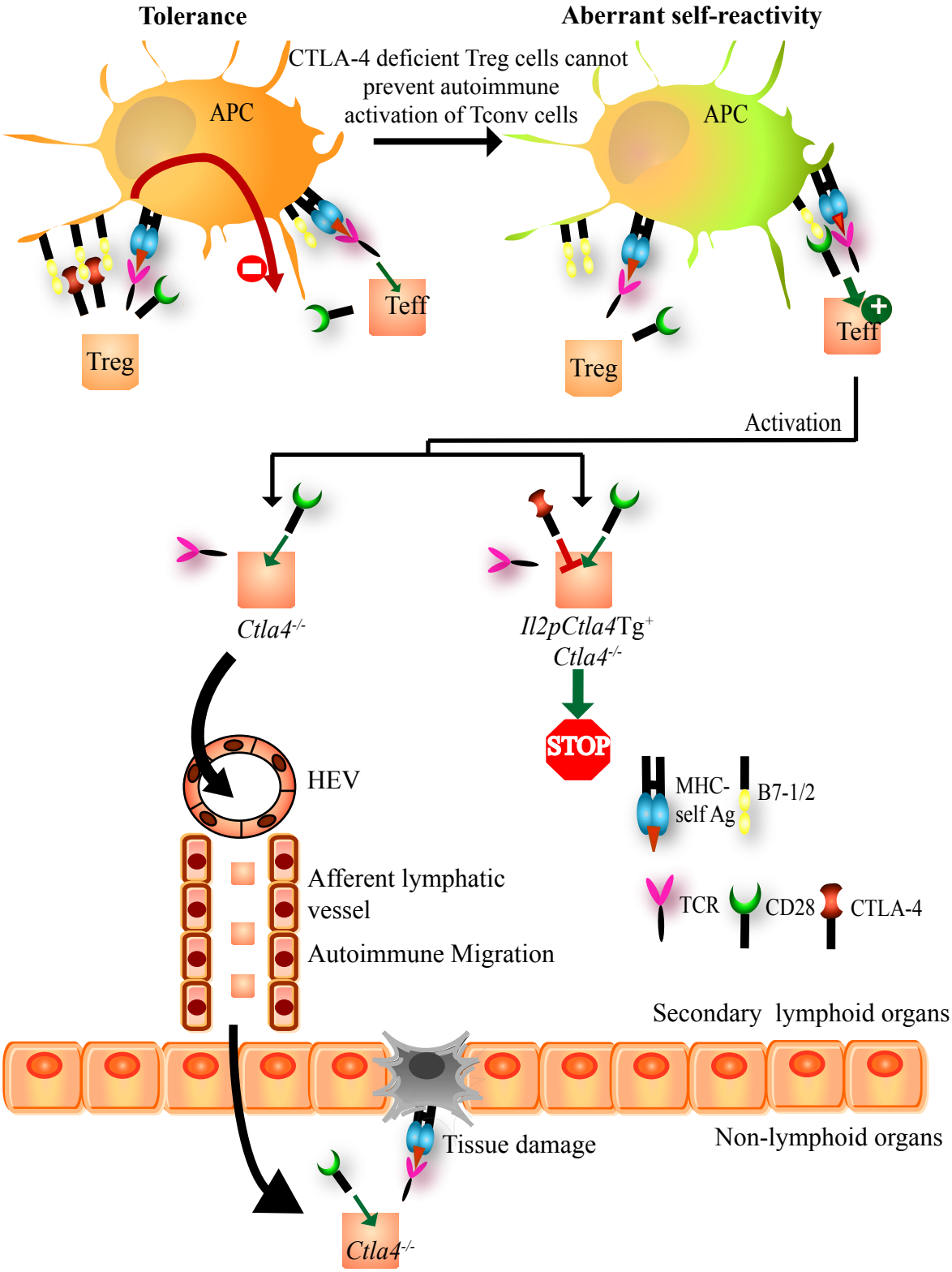

Figure IV-1: CTLA-4 controls aberrant self-reactivity and autoimmune tissue migration

Figure IV-1: CTLA-4 controls aberrant self-reactivity and autoimmune tissue migration

In a naïve mouse, CTLA-4 is primarily expressed on FOXP3^{+} Treg cells, with little to no expression on naïve conventional (Tconv) cells. Treg cells engage APCs that express low levels of B7-1/B7-2. Due to the higher affinity of CTLA-4 for B7-1/B7-2, they are preferentially ligated by CTLA-4 expressed on Treg cells. This delivers inhibitory signals into APCs that lead to the induction of immunosuppressive factors such as Indoleamine 2,3-dioxygenase (IDO) and TGFβ (*Top Left*). Modulation of APCs by Treg cells, in combination with the sequestration of B7-1/B7-2 molecules by CTLA-4, maintains peripheral tolerance, resulting in Tconv cells receiving only tonic TCR survival signals. In contrast, Treg cells lacking CTLA-4 are unable to condition APCs, and Tconv cells get aberrantly activated to self-antigen due to increased positive costimulation (*Top Right*). Once activated, however, an additional level of regulation is imposed on self-reactive Tconv cells by CTLA-4, which halts the migration of these T cells to non-lymphoid sites and prevents autoimmune pathology (*Middle*). As a result, while aberrantly-activated *Ctla4*$^{-/-}$ T cells can infiltrate tissues and cause their fatal destruction (*Bottom*), Tg*Ctla4* expression only in activated T cells prevents this.

proliferation and the 'burst size' of the T cell response, apoptosis of activated T cells and contraction of the immune response.

In addition to CTLA-4, we have identified Disabled-2 (DAB2) as another factor that is important for Treg cell function. In this study, we have shown that DAB2 is a FOXP3 target gene and its expression is restricted to $FOXP3^{+}$ Treg cells in peripheral lymphoid organs. DAB2 function in Treg cells is essential for *in vitro* and *in vivo* suppression of conventional T cell reactivity. However, at odds with this important role for DAB2 in Treg cells is the fact that *Dab2*-conditional knockout mice do not suffer from any pathology resulting from aberrant Treg cell function. In fact, deficiencies in *Dab2*-deficient Treg cells are only revealed when Treg cell function is tested in models involving homeostatic proliferation. For instance, in the cure of colitis model, naïve T cells are injected into $Rag^{-/-}$ mice where they proliferate and induce colitis. In this model, *Dab2*-deficient Treg cells are unable to prevent disease. Similarly, in a mixed bone marrow chimera system, Treg cells generated from *wt* BM can control the activation and proliferation of $Ctla4^{-/-}$ T cells (this system is described in Chapter II). However, in a similar assay, *Dab2*-deficient Treg cells fail to control lymphoproliferation and disease (data not shown). Finally, in preliminary experiments, we have sub-lethally irradiated *Dab2*-conditional knockout mice to induce homoeostatic proliferation. Recent data from this experiment shows that while *wt* LMC mice remain healthy at 3 weeks post-irradiation, *Dab2*-conditional knockout mice are sick and are beginning to lose weight. The data are too preliminary to make any conclusions, but they support the previous observations that *Dab2*-deficient Treg cells may have a specific role in regulating homeostatic proliferation induced loss of tolerance of naïve T cells.

While *Dab2* is expressed in FOXP3^{+} Treg cells in the thymus and periphery, this expression is relatively lower compared to *Dab2* expression in thymic precursor cells (**Figure III-1B**). One explanation for this is that the signals that regulate *Dab2* expression in Treg cells and thymic precursor cells are probably different. *Dab2* expression is most likely regulated by FOXP3 in peripheral lymphocytes, while *Dab2* expression in thymic precursor cells is clearly FOXP3 independent. Another possibility is that *Dab2* expression may be restricted to a subset of FOXP3^{+} Treg cells that might have a specific role in the regulation of homeostatic proliferation described previously. Context-dependent regulation by Treg cells is not a new concept, and it has been shown that Treg cells utilize different effectors to control different aspects of tolerance. For instance, IL-10 is critical for the regulation at mucosal surfaces, while CTLA-4 is critical for inhibiting the initiation of T cell activation to self-antigen. However, so far, the existence of subsets of FOXP3^{+} Treg cells with distinct functionality has not been reported. To begin to address this, we have analyzed *Dab2* expression in Treg cells separated on the basis of expression of specific surface markers. In preliminary experiments, we sorted Foxp3eGFP^{+}CD44^{hi}CD62L^{hi} and Foxp3eGFP^{+}CD44^{lo}CD62L^{hi} cells from *Foxp3*eGFP reporter mice. Real-time PCR analysis revealed that *Dab2* expression was higher in the more 'activated' CD44^{hi}CD62L^{hi} Treg cell population (data not shown). We are currently testing the significance of this difference in *in vitro* and *in vivo* assays.

A distinctive phenotype of *Dab2*-deficient conventional T cells is their altered responsiveness to TGFβ signals that regulate the conversion to FOXP3^{+} Treg cells. However, while the generation of the Treg cell lineage is impaired, *Dab2*-deficient

conventional T cells can respond to TGFβ signals that regulate the conversion to the Th17 lineage. The molecular intermediates of the TGFβ signaling pathway that regulate differentiation to the FOXP3 versus the Th17 lineage are just beginning to be elucidated. Signaling from the TGFβR can activate both the SMAD pathway as well as a MAPK pathway leading to JNK phosphorylation. Although the SMAD3 and SMAD4 pathways appear to be essential for the conversion of conventional T cells to the $FOXP3^+$ Treg cell lineage (280), whether the MAPK pathway of signal transduction from the TGFβR is differentially required for the commitment to the Th17 cell lineage is unknown. DAB2 functions as an adaptor molecule in both these pathways (248, 249). Therefore, one likely explanation for the ability of *Dab2*-deficient conventional cells to convert to Th17 cells could be because of an intact MAPK pathway that may be required for Th17 cell generation and an impaired SMAD pathway that is essential for $FOXP3^+$ Treg cell generation. It remains to be determined if indeed the MAPK pathway of TGFβ signal transduction is intact in *Dab2*-deficient T cells.

The role of retinoic acid (RA) in regulating Treg cell function and effector T cell differentiation is beginning to be unraveled. Of interest is that RA can lift the specific defect of *Dab2*-knockout conventional T cells to convert to $FOXP3^+$ Treg cells in the presence of TGFβ. Whether this is due to augmentation of TGFβ signals or by a TGFβ independent signaling pathway remains to be determined. Further, there are some indications that retinoic acid may by itself serve as a morphogen. The role of retinoic acid and DAB2 in the development of thymocytes and peripheral homoestasis is currently being investigated.

One function of DAB2 is to serve as an adaptor protein to help SMAD proteins associate with TGFβRII. In macrophage cell lines and epithelial cells, DAB2 functions as a positive regulator of TGFβ signals. Whether DAB2 performs similar functions in precursor cells in the thymus and Treg cells in the periphery is not known. One indication that DAB2 may have different effects in T cells is the fact that there is increased SMAD phosphorylation in the absence of DAB2. The function of DAB2 as an interpreter of TGFβ signals is being tested in *in vitro* over-expression studies.

Based on the data collected so far, we propose a model of DAB2 function in which *Dab2* expression in thymic precursor cells programs the TGFβ responsiveness of mature T cells to TGFβ in the periphery (**Figure IV-2**). Further, DAB2 is a critical effector of Treg cell function and one mechanism by which DAB2 may regulate this is by the formation of Gap-Junctions and facilitating transfer of cAMP.

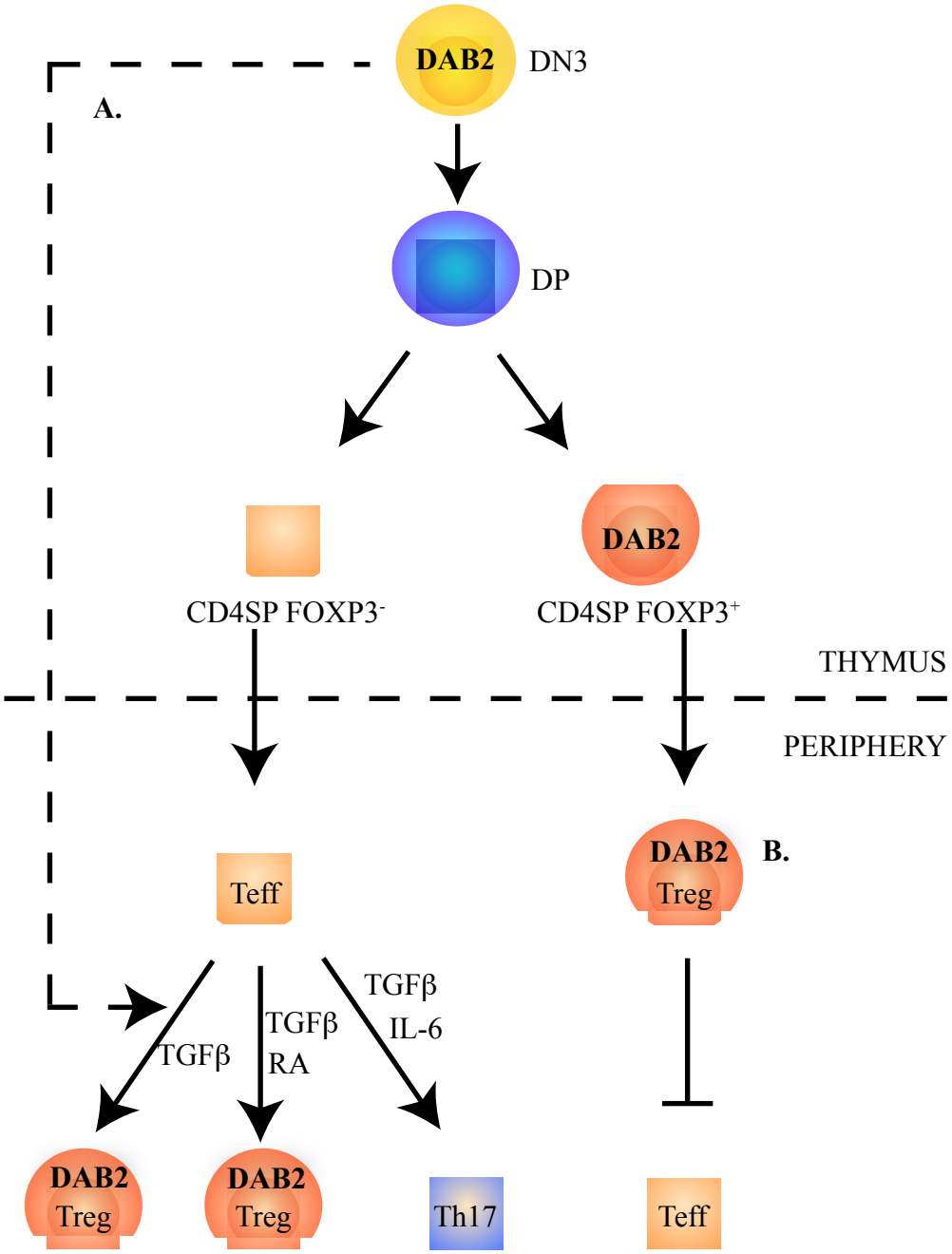

Figure IV-2: DAB2 programs TGFβ responsiveness of effector T cells in the thymus and controls Treg cell function

Figure IV-2: DAB2 programs TGFβ responsiveness of T cells in the thymus and controls Treg cell function

A. DAB2 expression in DN3 ($CD4^-CD8^-CD25^+CD44^-$) cells in the thymus is necessary to program TGFβ responsiveness of mature T effector (Teff) cells in the periphery. In the absence of DAB2, the differentiation of Teff cells to $FOXP3^+$ Treg cells in the presence of TGFβ is inhibited. However, retinoic acid (RA) can relieve this defect and restore normal TGFβ responsiveness.

B. DAB2 expression in Treg cells is important for Treg cell control of Teff cell function. In the absence of DAB2, Treg cells are not functional.

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